APS Conference

The Cellular and Molecular Biology of Membrane Transport

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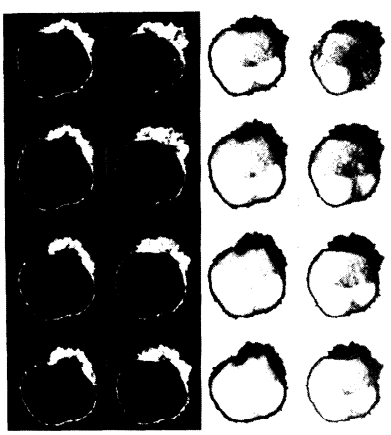

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The Cellular and Molecular Biology of Membrane Transport

Sessions with Contributed Abstracts

Thursday, November 5

Friday, November 6

Wednesday, November 4	Thursday, November 5	Friday, November 6	Saturday, November 7
Registration and Reception	Morning Symposium 9:00 am–Noon Structure and function of membrane ion channels Douglas Eaton	Morning Symposium 9:00 am–Noon Structure and function of membrane carriers Doug Fambrough	Morning Symposium 9:00 am–Noon Cellular modulation of membrane transport Lazaro Mandel
Evening Lecture 8:00–9:00 pm Molecular properties of voltage-gated ion channels William Catterall	Afternoon Posters 1:00–4:00 pm Poster Sessions Chloride and other anion channels. Sodium, potassium and other channels. Calcium transport and regulation of intracellular calcium.	Afternoon Posters 1:00–4:00 pm Poster Sessions Transport of sugars, amino acids and other organic substrates. Cell volume regulation. Membrane ATPases and other ion transport systems. Systemic and integrative physiology.	Afternoon Symposium 1:00–4:00 pm Post-translational regulation on ion channels Jack Kaplan
	Evening Symposium 7:30–10:00 pm Structure and function of membrane channels II: Molecular approaches to the properties of calcium channels William Agnew	Banquet 6:00 pm—Reception 7:00 pm—Dinner	
		Evening Lecture 8:00–9:00 pm The ins and outs of lactose permease of *Escherichia coli* Ron Kaback	

4.1

FUNCTIONAL CHARACTERIZATION OF ρ_1 AND ρ_2, GABA RECEPTOR SUBUNITS. T.-L. Wang*, G. R. Cutting*, and W.B. Guggino*, Depts. of Physiology and Pediatrics., The Johns Hopkins Univ., School of Medicine, Baltimore, M.D. 21205.

The $GABA_A$ receptor is a multi-subunit Cl^- channel found in brain. Recently, two new, ρ_1 and ρ_2, subunits were cloned and sequenced which display considerable sequence homology to $GABA_A$ receptors. ρ_1 injected into *Xenopus* oocytes functions as a GABA-activated Cl^- channel with distinctive pharmocological properties. Our current aim was to compare ρ_1 and ρ_2 using *Xenopus* oocytes and a two electrode voltage clamp. GABA (5 μM)-induced currents were detected in oocytes injected either with 10 ng of ρ_1 or ρ_2 RNA, but not with water (n=6). The I-V relationships suggested a higher conductance for ρ_1 than for ρ_2 (n=5). Coexpression of ρ_1 and ρ_2 resulted in GABA-activated currents of greater magnitude than observed when they were expressed singly suggesting that the two subunits can interact. The ρ_1 expressed current displayed more desensitization to continued exposure to 5 μM GABA than ρ_2. After 70 seconds, the current amplitude diminished by 35% (n=4) for ρ_1 whereas, ρ2 was reduced by only 5% (n=6). The pharmacology of the two subunits was similar. 5 uM picrotoxin applied with 5 μM GABA, reduced the currents for ρ_1 and ρ_2 to 50% (n=6) and 60% (n=7) of control, respectively. Currents were reduced to 20% and 28% of control, respectively by 50 μM, $ZnCl_2$, which was reversed by adding EDTA. ρ_2, like ρ_1, was not sensitive to bicucullin, hexobarbital, or benzodiazepam. Our data suggest that the ρ_1 and ρ 2 represent a unique class of GABA receptor Cl^- channels.

4.2

ROLE OF G-PROTEINS IN SIGNAL TRANSDUCTION OF EPIDERMAL GROWTH FACTOR RECEPTORS IN PANCREATIC ACINAR CELLS.
Albrecht Piiper*, Danuta Stryjek-Kaminska* and Stefan Zeuzem* (SPON: J. Piiper) Dep. of Internal Medicine, Univ. of Frankfurt, 6000 Frankfurt/Main 70, FRG

Cholecystokinin (CCK) stimulates pancreatic enzyme secretion by increasing acinar inositol 1,4,5-trisphosphate (IP3) content in a process that involves heterotrimeric GTP-binding proteins (G-proteins). A recent study indicates that the Cl^- conductance in isolated zymogen granules from rat pancreatic acini is closely associated with acinar enzyme and electrolyte secretion and that epidermal growth factor (EGF) inhibits the CCK-induced Cl^- conductance in zymogen granules. Here, we investigated the role and the mechanism of action of EGF on basal and CCK-induced IP3-production in digitonin-permeabilized rat pancreatic acini. The data show that EGF (17 nM) had no effect on the basal IP3 level. However, it caused a significant IP3-accumulation in the presence of the G-protein inhibitor GDP (1 mM). By contrast, it inhibited CCK (100 nM)-induced IP3-production at five seconds after beginning of the incubation approximately tenfold. GDP reduced the inhibitory effect of EGF on CCK-induced IP3-production. These results suggest a dual mode of action of EGF in acinar signal transduction: EGF activates phospholipase C by a G-protein-independent process, whereas it inhibits CCK-induced activation of phospholipase C by activating CCK receptor coupling G-proteins.

4.3

CYCLIC GMP ACTIVATES CFTR CHANNELS EXPRESSED IN OOCYTES.
Stephen K Sullivan, Rong Schick, Richard J Gregory+, Luis B Agellon and Michael Field. Columbia University, New York, NY 10032 and +Genzyme Corporation, Framingham, MA 01701

Cyclic nucleotide phosphodiesterase (PDE) inhibitors like theophylline and 3-isobutyl-1-methylxanthine activate CFTR by increasing cAMP. These compounds also increase cGMP, but the effects of cGMP independent of cAMP have not been examined. When we injected cGMP (0.5-50 μM) into oocytes expressing CFTR from recombinant mRNA, we observed a gradual increase in current that plateaued after 3-4 minutes at about one-half the magnitude observed in oocytes stimulated by cAMP. One possible mechanism for cGMP activation of CFTR is by increasing cAMP via the so-called cGMP-inhibitable cAMP PDE. Another possible mechanism is via activation of cGMP-dependent protein kinase (PKG). These possibilities were tested using the protein kinase inhibitor H-8. H-8 (20 μM) reduced cAMP-mediated activation of CFTR by 44% but had no effect on cGMP-mediated activation. Protein kinase inhibitor (PKI, 100 nM), a 20 residue peptide highly selective for inhibition of cAMP-dependent protein kinase (PKA), reduced cAMP-mediated activation by 76% but had no effect on cGMP-mediated activation. KT-5823 (1 μM), a specific inhibitor of PKG, had no effect on CFTR activation by either cyclic nucleotide. Cyclic GMP also activated mutant CFTR carrying the phenylalanine 508 deletion. We conclude that cGMP activates CFTR by a mechanism independent of PKA and PKG, perhaps by direct interaction with the channel.

4.4

LOCALIZATION OF CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR IN INTESTINAL GOBLET CELLS. H.V. Carey, C.M. Fuller and D.J. Benos. Department of Comparative Biosciences, University of Wisconsin, Madison WI 53706 and Department of Physiology and Biophysics, University of Alabama, Birmingham AL 35294.

The cystic fibrosis transmembrane regulator (CFTR), the gene product defective in cystic fibrosis, is a member of a family of ATP binding proteins that act as unidirectional solute pumps. Although CFTR has been primarily localized to apical regions of a variety of epithelial cell types, its occurrence within cells has also been described. We report here the localization of CFTR within goblet cells in the piglet small intestine. Monospecific rabbit polyclonal antibodies raised against a synthetic peptide corresponding to residues 785-797 of the R-domain of CFTR were used to localize immunocytochemically the protein in thick (6 μm) sections of piglet jejunum using light microscopy. CFTR was detected almost exclusively in goblet cells in the crypt region, and to a lesser extent in villus cells. The protein was localized intracellularly, basal to the mucin granules. Immunoreactive staining patterns were completely blocked by prior incubation of anti-CFTR antibodies with peptide 785-796. On Western blots the antibodies recognized a protein that migrated at an apparent molecular mass of 170-180 kDa. In keeping with the immunocytochemical findings, specific bands were strongest in cytosolic fractions of mucosal homogenates compared with purified brush border membranes. These results suggest that CFTR may be involved in intracellular solute transport in intestinal goblet cells. Supported by funds from the University of Wisconsin School of Veterinary Medicine, and NIH grant DK42017.

4.5

POWER DENSITY SPECTRUM AND SINGLE CHANNEL ACTIVITY ASSOCIATED WITH CFTR MEDIATED Cl^- CURRENTS. E.H. Larsen*, S.E. Gabriel*, J. Fullton*, R.C. Boucher*, E. Price*, and M.J. Stutts* (SPON: C.W. Davis). Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7020, USA.

Spodoptera frugiperda (Sf9) cells were infected with human CFTR by the Baculovirus vector expression system. With 150 mM external Cl^-, 40 mM Cl^- in the cytosolic-like pipette solution (E_{Cl} = -33 mV), and $TRIS^+$ as the major cation, whole cell membrane currents reversed at -30±2 (CFTR, n=18), and -34±2 mV (uninfected, n=13), and obeyed the GHK-equation with P_{Cl} = 3.4±0.5 (CFTR) and 2.4±0.3 (uninf.) 10^{-9} $cm^3/(s\ cell)$. Cl^- currents (I_{Cl}) of CFTR-infected cells, only, were activated by forskolin (FSK), and when clamped at V-E_{Cl} = 73 mV, stationary current fluctuations exhibited large CFTR-dependent variance, σ^2 (PA^2) = 42.0±2.5 (CFTR, FSK) 6.64±0.29 (uninf., FSK), which were eliminated at V = E_{Cl} (0.811±0.005 pA^2, CFTR, FSK, bandwidth 1 kHz). The I_{Cl}-power density spectrum was resolved in 3 Lorentzian components with corner frequencies of about 0.5, 10, and 50 Hz, respectively. In cell attached patches (CFTR, FSK) we recorded 8-11 pS channels with wave-like opening patterns and low-frequency transitions of larger conductive states (30-40 pS), which reversed at estimated E_{Cl}. Large conductive states lasted for >20 s and were built up and degraded in step-like fashion. We conclude that CFTR-mediated and FSK-activated I_{Cl} does not result from a single population of small channels with first order transition kinetics. (NIH HL42384, DNSRC 11-9270).

4.6

A PATCH-CLAMP STUDY OF EPITHELIAL ION CHANNELS RECONSTITUTED INTO GIANT LIPOSOMES. Marek Duszyk*, Andrew S. French* and S.F. Paul Man. Univ. Alberta, Depts. Medicine and Physiology, Edmonton, Alberta T6G 2C2 CANADA

Reconstitution of ion channels into artificial membranes allows the identification of proteins associated with given ion channels and can provide information about the minimal components required for channel function. To study ion channels in the apical membranes of cultured CFPAC-1 cells, membrane proteins were isolated and incorporated into giant liposomes for patch clamp recording. An apical plasma membrane fraction was prepared by magnesium precipitation/differential centrifugation. Proteins were solubilized with 10 mM CHAPS, suspended at ~2mg/ml, and stored at -80°C.

Giant liposomes were formed by a dehydration-hydration method. L-α-lecithin (10 mM), apical membrane proteins, and 10 mM CHAPS (5:3:2 volume ratio) were dialysed, spun and suspended in saline containing 5% ethylene glycol. The suspension was dehydrated for 3h in a $CaCl_2$ desiccator, and then rehydrated. Giant liposomes were observed the following day.

Ion channels were characterized using the excised inside-out configuration. Three types of anion channels with conductances of 24, 140, and 380 pS were observed. Cation permeable channels had a conductance of about 68 pS.

4.7

CHLORIDE CHANNELS IN THE RENAL PROXIMAL TUBULE. Alan S. Segal* and Emile L. Boulpaep, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510.

Using individual, polarized *Ambystoma* renal proximal tubule cells we have identified linear 10 pS chloride channels on the basolateral membrane (BLM). The open probability of this channel increases with depolarization, and its selectivity sequence is $I^- > Br^- > Cl^- >> F^- > Aspartate^-$ and $Cl^- >> Na^+$. With equal pipette and bath chloride, whole-cell patch-clamp records show a macroscopic chloride conductance reversibly stimulated by either 10 μM forskolin or 0.5 mM dibutyryl-cAMP. The same agents activate chloride channels in cell-attached patches on the BLM. In excised inside-out patches, the channels are stimulated by addition of the catalytic subunit of protein kinase A. Subsequent deactivation by phosphatase supports the hypothesis that the channel is regulated via phosphorylation. A 10 pS chloride channel on the BLM is also sensitive to stretch. Negative pressure of 2-4 cm H_2O applied to cell-attached patches activates chloride channels. The channels promptly close when the pressure is released, but immediately open again if stretch is reapplied. The number of open channels increases with rising negative pressure. Insensitivity of excised patches to stretch suggests that an intact cytoskeleton and/or cytosolic factors are required for stretch-activation of these chloride channels. We conclude that the basolateral membrane of single polarized proximal tubule cells contains linear 10 pS chloride channels that are activated by stretch and cAMP. We postulate that control of basolateral chloride channels regulates both cell volume and transcellular reabsorption of chloride in the proximal tubule.

4.8

cAMP-DEPENDENT REGULATION OF CHLORIDE PERMEABILITY IN PRIMARY CULTURES OF MAMMALIAN COLONOCYTES J. Sahi*, J.L. Goldstein*, L.N. Schmidt*, T.J. Layden* and M.C. Rao. Depts. of Physiology & Biophysics and Medicine, Univ. of Illinois at Chicago, Chicago, IL, 60612.

We have developed a model of short-term primary cultures of human rectal and rabbit distal colonic epithelial cells and used these cells to evaluate the regulation of Cl permeability. Cl transport was assessed in 24-hour cultured colonocytes using the Cl-sensitive fluorescent probe: N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE). In both human (n=4) and rabbit (n=5) colonocytes, secretagogues known to stimulate Cl secretion in intact colonic epithelia including 1μM forskolin, 100 μM 8-Br-cAMP, 1 μM PGE_1 and 10μM histamine increased Cl influx. Depending on the secretagogue, the stimulated influx rates were 1.7 - 6.6-fold greater than basal. The Cl channel blocker, DPC (5 μM) inhibited basal Cl influx by ≈ 70% and secretagogue-activated Cl influx by ≈ 50-70%. Phorbol dibutyrate (1μM), also stimulated Cl influx, but had a greater effect in human (530%) than in rabbit (240%) colonocytes. In rabbit colonocytes, the phosphatase inhibitor okadaic acid (1μM) caused a 2.5-fold increase in Cl influx and its effects were additive to those of forskolin. These results demonstrate that rabbit and human colonocytes in culture possess DPC-sensitive Cl channels and other, yet to be identified, Cl transporters. They also demonstrate that the Cl permeability in these cells is cAMP-activatable and involves a phosphorylation/dephosphorylation mechanism. These transport properties are similar to those of intact epithelia. The short-term culture therefore provides a good model to study cellular and molecular basis of regulation of ion transport in the human colon. (Supp. by CFF & NIH-HL07692).

4.9

A SUBPICOSIEMENS Cl^- CHANNEL IS ACTIVATED BY FORSKOLIN IN THE APICAL MEMBRANE OF POLARIZED HT-29 CELLS. Horst Fischer, Beate Illek, and Terry E. Machen, Molecular and Cell Biology, University of California, Berkeley, CA 94720.

Noise analysis of transepithelial currents (I_{SC}) was performed to investigate the apical Cl^- exit step in HT-29/B6 monolayers. Cells were grown to confluency on permeable supports and were measured in Ussing chambers under voltage clamp conditions. Unstimulated I_{SC} was close to zero and transepithelial resistance was R_T=302±27 Ω·cm². In order to get access to the apical membranes of the cells, the basolateral membrane was permeabilized with amphotericin B, and a serosal-to-mucosal Cl^- gradient (150:7.5 mM) was applied to induce Cl^- current in the secretory direction. Under these conditions R_T dropped to 261±22 Ω·cm² (p<0.02) indicating a successful permeabilization of the basolateral membrane. I_{SC} was 22±2 μA/cm², and a very low frequency Lorentzian noise component emerged in the current noise spectra with a corner frequency f_c=1.2±0.2 Hz (n=14). Stimulating this preparation with forskolin increased I_{SC} to 77±13 μA/cm² and decreased R_T to 172±28 Ω·cm² indicating activation of an apical Cl^- conductance. The power of the Lorentzian increased significantly from 275±53 to 592±69·10^{-21} A^2·s/cm² (p<0.01), and f_c remained unchanged (2.3±0.8 Hz, n.s.) indicating an increased number and/or increased open probability of the underlying slow gating Cl^- channel. Estimation of single channel conductance from Lorentzian noise revealed a Cl^- channel smaller than 1 pS. We conclude that a subpicosiemens, high abundance Cl^- channel in the apical membrane of HT-29/B6 cells is responsible for Cl^- secretion during forskolin-stimulation. (Supported by DFG, NIH, CF Foundation, and CF Research, Inc.).

4.10

HYPOSMOTIC STRESS ACTIVATES CALCIUM-DEPENDENT CHLORIDE CHANNELS IN ISOLATED RAT LACRIMAL ACINAR CELLS. T. Kotera*, P. D. Brown* and R.M. Case* (SPON: E. M. Wright). Dept. of Physiol. Sci., Univ. of Manchester, Manchester M13 9PT, UK

Cl^- channels play an important role in fluid secretion in lacrimal acinar cells. Recent work has identified volume-activated Cl^--channels in some secretory epithelia. In this study we have investigated the effect of hyposmotic stress on Cl^--channel activity in isolated rat lacrimal acinar cells. Whole-cell Cl^--current was monitored at a holding potential of -60 mV. The pipette solution contained (mM): 145 N-methyl-D-glucamine (NMDG)-Cl, 2 $MgCl_2$, 0.5 EGTA, 5 HEPES, and the bath (mM): 145 NMDG-Cl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES. Perfusing a hyposmotic solution (80 % of control osmolarity) into the bath caused a gradual increase (1-2 min.) in the Cl^- current, which was reversed on return to the control solution. The profile of this current showed time- and voltage-dependent activation. The I-V relationship showed outward-rectification. The current increase was not observed in the absence of intracellular Ca^{2+} (10 mM EGTA in the pipette solution). It was also inhibited by extracellular Ca^{2+}-free solutions ($CaCl_2$ replaced by 0.5 mM EGTA), or in the presence of 20 μM Gd^{3+} in the bath solution. These results suggest that hyposmotic stress activates Ca^{2+}-dependent Cl-channels. These channels are probably the same as those activated by secretagogue stimulation. Hyposmotic stress appears to activate a Gd^{3+}-sensitive Ca^{2+} influx pathway, suggesting the involvement of Ca^{2+}-permeable stretch-activated channels.

4.11

REGULATION OF CHLORIDE CURRENT IN OSTEOCLASTS. Melanie E.M. Kelly*, S. Jeffrey Dixon & Stephen M. Sims. Dept. Physiology & Div. Oral Biology, The University of Western Ontario, London, Canada and Dept. Pharmacology, Dalhousie University, Halifax, Canada

We characterized chloride currents in freshly isolated rabbit osteoclasts using patch clamp methods. An outward current, distinct from the inwardly rectifying K^+ current previously reported (Kelly et al., *J. Memb. Biol.* 126:171, 1992) was apparent in 40 to 50% of cells. The outward current persisted when all K^+ currents were blocked and was inhibited by stilbene disulfonates and niflumic acid, chloride channel blockers. The blocked current reversed direction close to the chloride equilibrium potential. In those osteoclasts in which outward current was not initially apparent, exposure to hypoosmotic extracellular solution resulted in activation of outward current. The induced current was a Cl^- current, based on its dependence on the chloride gradient and sensitivity to Cl^- channel blockers. The hypoosmotically induced current was not dependent on extracellular Ca^{2+} since outward current could be activated in Ca^{2+}-free solution containing 0.2 mM EGTA. When studied in current clamp, hypoosmotic stimulation caused depolarization from ≈-75 mV to -5 mV. Cl^- currents were recorded in the cell-attached patch configuration at positive potentials with slope conductance of 19±5pS (n=5). Single Cl^- channel currents were also observed following hypoosmotic stimulation. We conclude that osteoclasts express Cl^- channels that may play a role in cell volume regulation. Cl^- channels may also provide conductive pathways for dissipating the potential difference that arises from electrogenic proton transport across the ruffled membrane of the osteoclast. Supported by The Arthritis Society and the Medical Research Council of Canada.

4.12

PROPERTIES OF A PROTEIN KINASE C-ACTIVATED CHLORIDE CURRENT IN CARDIAC VENTRICULAR CELLS. Kenneth B. Walsh* (SPON: P.D. Watson). Univ. of South Carolina, Columbia, SC 29208.

The whole-cell arrangement of the patch clamp technique was used to examine the effect of protein kinase C (PKC) on ion channels in isolated guinea pig ventricular cells. In the presence of appropriate internal and external solutions to reduce contamination from Na^+, Ca^{2+} and K^+ currents, application of phorbol 12,13-dibutyrate, or internal dialysis of cells with partially purified PKC, activated a time-independent background current. Alteration of the Cl^- equilibrium potential, brought about through changes in external and internal Cl^- concentrations, shifted the reversal potential for the background current in manner expected for a Cl^--selective ion channel. When the Cl^- concentration inside the cell was less than that outside (i.e. 70 mM inside/146 mM outside) the current normally displayed outward rectification. Based on reversal potential measurements, the permeability sequence for the channel was determined to be $I^- > Br^- \approx Cl^-$. Although insensitive to 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS), the current could be inhibited by high concentrations of monocarboxylic acid compounds. It is concluded that cardiac ventricular cells contain PKC-activated Cl^- channels.

4.13

Excitation-contraction uncoupling of rat ventricular myocardium due to chloride ion depletion. Warren Tse,* (Spon: PYD Wong) Department of Physiology, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, NT, Hong Kong.

Using voltage clamp technique, Cl channels are identified in the ventricular myocytes (Ehara & Ishihara 1990). However, the function of I_{Cl} in myocardium remains to be determined. The present study is to report a novel phenomenon in that uncoupling of the excitation-contraction (E-C) of isolated rat hearts occurs in absence of $[Cl^-]_o$. Langendorff rat heart preparations perfused with Krebs-Henseleit solution (KHS) were employed. The heart was paced electrically at 270 pulses per min at the right ventricle. A polygraph was employed to record the ECG (lead II) and force of ventricular contraction (VC). The change in cardiac functions were determined in 1). Cl^--free KHS, the Cl^- was substituted by SO_4^{2-}, and 2) the presence of a Cl^- channel blocker, dephenylamine-2-carboxylate (DPC) at 3.0×10^{-4} M. In 7 experiments, the perfusion of Cl^-free K-H-S resulted in significant suppression of the VC to 28 SD ± 18 % of the control.($P<0.001$). In another group of 10 experiments, DPC also suppressed the the VC to 26 SD ± 15% of the control ($P<0.001$). Contrarily, the amplitude of the QRS waves of the ECG was increased when exposed to Cl-free KHS to 231 SD ± 57% of the control; however, no significant change on this parameter occurred when exposed to DPC. In summary, the present findings strongly indicate that $[Cl^-]_o$ plays an important role in the E-C coupling of the myocardium because its depletion results in E-C uncoupling. It is possible that the Cl^- influx affects the $[Ca^{2+}]_o$ influx which plays an important role in the E-C coupling of the myocardium.

4.14

INTERACTION OF RENAL P-GLYCOPROTEIN WITH VARIOUS DRUGS AND A HYDROPHOBIC COMPONENT IN URINE. Jeffrey H.M. Charuk* and Reinhart A.F. Reithmeier* (SPON: M. Silverman). MRC Group in Membrane Biology, Department of Medicine, University of Toronto, Toronto, Ontario, Canada, M5S 1A8.

To gain an understanding of P-glycoprotein's transport function in kidney and its potential role in drug-induced nephrotoxicity we have studied its interaction with various drugs and a hydrophobic component in rat urine that specifically inhibits P-glycoprotein activity. Rat kidney brush border membranes were photolabelled with [^{3}H]-azidopine and P-glycoprotein immunoprecipitated with a rabbit polyclonal antibody to a human mdr-1 fusion protein. Whereas digoxin failed to inhibit photolabelling, other drugs known to interact with P-glycoprotein in multidrug-resistant cells such as vinblastine and verapamil blocked photolabelling. Very low concentrations of cyclosporin A and the hydrophobic rat urine extract blocked photolabelling of renal P-glycoprotein also. Our current hypothesis is that various drugs by virtue of their ability to bind with high affinity to P-glycoprotein will competitively inhibit the urinary secretion of an endogenous substrate. Purification of this hydrophobic urine component and subsequent determination of its cytotoxic properties will greatly enhance our understanding of the mechanisms involved in the nephrotoxic action of important chemotherapeutic agents such as cyclosporin.

Supported by the MRC and Kidney Foundation of Canada.

SODIUM, POTASSIUM AND OTHER CHANNELS

5.1

A BACKGROUND Na^+ CONDUCTANCE IS ESSENTIAL FOR THE EXPRESSION OF SPONTANEOUS DEPOLARIZATIONS IN PITUITARY CELLS. Steven M. Simasko. SUNY at Buffalo, Buffalo, NY 14214.

Lactotrophs express spontaneous, Ca^{2+}-dependent depolarizations. The Ca^{2+} influx during these depolarizations may underlie the high rate of spontaneous secretion observed in these cells. The reason these cells depolarize to the threshold for activation of voltage-dependent calcium current (VDCC) has not been established. In this study GH_3 cells, a model pituitary cell line, were used to test the hypothesis that a background Na^+ conductance provides this depolarizing drive. Voltage- and current-clamp experiments were performed with the use of the perforated patch configuration of the patch clamp technique. Replacement of bath Na^+ with choline, Tris, or N-methyl-D-glucamine (NMDG) caused a prompt and reversible 20-30 mV hyperpolarization and cessation of spontaneous activity. This hyperpolarization is not explained by effects on conductances previously described in GH_3 cells. Tetrodotoxin (0.5 μM) had no effect. Replacement of bath Na^+ either caused no change (NMDG) or a decrease (10-25%, Tris and choline) in K^+ current. Replacing bath Na^+ did cause a slight decrease (10- 15%) in VDCC, however, inhibition of VDCC with nifedipine (1.0 μM), although halting spontaneous activity, led to a slight baseline depolarization (5 mV). A background Na^+ current (10-20 pA at -80 mV) can be directly measured. These results demonstrate that a background Na^+ conductance provides the depolarizing drive necessary to activate VDCC which leads to spontaneous regenerative depolarizations.

5.2

REGULATION OF SODIUM CURRENT BY DIBUTYRYLADENOSINE 3':5'-CYCLIC MONOPHOSPHATE AND PHORBOL 12,13-DIBUTYRATE IN *IN VITRO* GENERATED CARDIAC CELLS. Jorge Arreola and Ted Begenesich. University of Rochester, Rochester, NY 14642.

Pluripotent murine embryonal carcinoma cell line (P19) was *in vitro* differentiated with dimethyl sulfoxide and retinoic acid to generate cardiac- and neuron-like cells. Whole cell sodium current was recorded at room temperature from dissociated cardiac- and undissociated neuron-like cells. Sodium current activates around -40 mV with a maximum at -10 to 0 mV of membrane potential. Cardiac- and neuron-like sodium channels half inactivation was found at -80.6 ±3.7 mV and -45.9 ± 0.8 mV, respectively. Cardiac-like sodium channels were less sensitive to tetrodotoxin (TTX) than neuron-like channels, Kd_s 0.65 µM and 8.6 nM, respectively. Isoproterenol (1 µM) decreased the amplitude of the cardiac-like sodium current. Propranolol partially inhibited this effect. N^6,2'-O-Dibutyryladenosine 3':5'-cyclic monophosphate (DBcAMP) (15 µM) decreased the amplitude of the cardiac-like sodium current by 22.9 ± 7.4 %, and slowed the activation and decay of the sodium current. However, the steady-state inactivation was not significant affected by DBcAMP. It was also founded that DBcAMP required ATP to affect sodium channels. Protein kinase C activator, Phorbol 12,13 dibutyrate (PDBu) (50 nM), gradually decreased the amplitude of cardiac-like sodium current to 57.7 ± 9.8% of the control value in 30 minutes. PDBu significantly slowed the activation and inactivation of the cardiac-like sodium channels whereas the steady-state inactivation was unchanged. Peptide protein kinase C inhibitor (100 µM) in the patch pipette solution abolished the effect of 50 nM PDBu on the amplitude, activation and inactivation of sodium currents in the cardiac-like cells. These results suggest that the TTX-resistent sodium channels expressed by an *in vitro* generated cardiac-like cells are modulated by protein kinases A and C.

5.3

STRAIN DIFFERENCES IN AMILORIDE SUPPRESSION OF TASTE NERVE RESPONSES TO NACL AND KCL. M. M. Mineart* and R. J. Contreras. Program in Neuroscience, The Florida State University, Tallahassee, FL 32306-1051.

Fischer 344 and Sprague-Dawley rat strains differ in their NaCl preference. Sprague-Dawley rats exhibit a strong taste preference to a broad range of NaCl concentrations, while Fischer 344 rats treat NaCl as aversive at all concentrations. Salt-sensitive taste receptor cells, on the anterior of the tongue in the rat, are innervated by the chorda tympani nerve(CTn). Taste reception and transduction of NaCl is thought to be mediated by the passive transport of Na^+ ions across the plasma membrane of taste receptor cells. The purpose of the present study was to determine whether the strain difference in NaCl preference is mediated by differences in number of amiloride-sensitive Na^+ channels on the taste receptor cells. We predicted that amiloride would suppress CTn responses to NaCl to a greater degree in Fischer 344 as compared to Sprague-Dawley rats. The magnitude of the tonic responses to various concentrations of NaCl mixed in 1, 10, or 100 uM amiloride were compared to the responses to the same NaCl concentrations used alone. Our initial observations indicate that amiloride had a greater inhibitory effect on CTn NaCl responses in Fischer 344 rats as compared to Sprague-Dawley rats. Unlike prior reports in the taste literature, amiloride also had a small inhibitory effect on the CTn responses to KCl in both strains. The present data are consistent with the hypothesis that the strain difference in NaCl taste preference is a function of the number of amiloride-sensitive ion channels. (Supported by NIH grant HL38630).

5.4

EFFECTS OF AMILORIDE ANALOGUES ON RAT LUNG SODIUM TRANSPORT. Barbara E. Goodman and Mark A. Reynen*. Dept. of Physiology/Pharmacology, University of South Dakota, School of Medicine, Vermillion, SD 57069.

Benzylamiloride (benzamil) is amiloride analogue which more specifically inhibits Na^+ channels and dimethyl amiloride (dimethyl) is analogue which more specifically inhibits Na^+/H^+ antiporters. We have begun investigating the effects of amiloride analogues on PS_{Na} for clearance from distal airspaces of rat lungs. Airspaces of isolated degassed rat lungs were lavaged and instilled with 3 ml Kreb's Ringer Bicarbonate solution (KRB) containing trace amounts of ^{22}Na, ^{14}C-sucrose, and FITC-labelled dextran. Samples of single-pass KRB perfusate were analyzed for appearance of tracers. Apparent permeability-surface area products (PS) were calculated based on adaptation of Fick's First Law of Diffusion for control period and after amiloride analogue was added to perfusate. PS_{Na} values are given as 10^{-4} ml/s. (* significantly different)

	Control PS_{Na}	Drug PS_{Na}
Benzamil(10^{-6} M)	2.94 (±0.2)	2.24 (±0.3)*
Benzamil(10^{-9} M)	3.68 (±0.6)	2.83 (±0.4)
Dimethyl(10^{-6} M)	3.06 (±0.3)	2.68 (±0.4)*
Dimethyl(10^{-9} M)	3.52 (±0.1)	2.90 (±0.1)*
Amiloride(10^{-6} M)	2.95 (±0.1)	2.79 (±0.1)
Amiloride(10^{-8} M)	3.38 (±0.5)	3.11 (±0.6)
Amiloride(10^{-3} M)	3.67 (±0.4)	2.32 (±0.5)*

Amiloride is least potent. PS_{Na} decreased 24% (benzamil) and 16% (dimethyl) at low concentrations. (Supported by NIH HL38310)

5.5

INSULIN STIMULATES EXOCYTOSIS AT THE APICAL MEMBRANE OF CULTURED RENAL EPITHELIAL CELLS. D.Erlij, P.de Smet* and W. Van Driessche. SUNY, Downstate Med. Ctr., Brooklyn, N.Y., 11203, and KUL, Leuven, B-3000, Belgium.

Insulin stimulates Na^+ transport in tight epithelia by increasing both apical membrane Na^+ permeability and serosal conductance. In nonepithelial cells insulin stimulates transport by promoting the exocytotic transfer of transporters from an intracellular pool to the surface membrane. To find whether an exocytotic process is also involved in the epithelial effects of insulin we assessed changes in membrane area by measuring membrane capacitance in epithelia grown from the renal cell line A6. We also determined the number of open Na^+ channels in the apical membrane by analysis of blocker induced fluctuation in short circuit current (I_{sc}). Insulin significantly increased effective capacitance (C_T) by 15% when administered to tissues incubated in either Na^+ containing apical solutions (n=11) or in apical solutions in which all Na^+ had been substituted by $NMDG^+$ (n=10). These measurements indicate that insulin increases apical capacitance (C_a) because when basolateral capacitance is much larger than C_a, as in A6 cells, C_a is nearly equal to C_T. In tissues incubated with apical Na^+ insulin increased I_{sc} by 96% while the number of open Na^+ channels in the apical membrane was increased by 116%. These results show that insulin increases both apical membrane area and the number of open Na^+ channels.

5.6

A HIGH-AFFINITY AMILORIDE BINDING SITE, DIFFERENT FROM THE APICAL Na+ CHANNEL.

O. Goldstein*, C. Asher*, P. Barbry*, E.J. Cragoe* Jr., W. Clauss*, M. Lazdunski*, and H. Garty. *Dept. of Membrane Research & Biophysics, The Weizmann Institute of Science, Rehovot 76100, Israel.*

Specific binding of [3H]-phenamil and [3H]-benzamil to chicken colon plasma membrane was investigated. A single population of sites with high affinity to phenamil (binding constant of 4.8 nM); benzamil (6.0 nM); and amiloride (19.8 nM), was identified. Matched comparison of [3H]-phenamil binding and $^{22}Na^+$ uptake inhibition, in the same membranes, revealed two major differences between this site and the site whose occupancy blocks the channel. First, 5-(N-ethyl-isopropyl) amiloride (EIPA) displaced specifically bound [3H]-phenamil at concentrations that are 10-100 fold lower than those needed to inhibit transport. Second, the rates at which [3H]-phenamil associated and dissociated from membranes, were considerably lower than the rates at which channels were inhibited and re-activated upon the addition and removal of phenamil. A similar site with high affinity to both amiloride and EIPA was measured also in membranes from toad bladder and A6 cells but not in chicken brain or heart. Low NaCl diet and a chronic administration of aldosterone largely increased channel activity but did not affect the abundance of [3H]-phenamil binding sites. We conclude that tight epithelia contain a major high affinity amiloride binding site different from the one whose occupancy blocks the Na+ channel. This site could be associated with a pool of non-conductive channels, or a totally unrelated protein. Supported by The Joseph Cohen Center for Biomembrane Research and The Ebner Family Biomedical Research Foundation, at The Weizmann Institute of Science.

5.7

GOSSYPOL ACTIVATES A SODIUM CONDUCTANCE IN EPITHELIAL CELLS. Andreas Jungwirth*, Thomas Weiger*, Anton Hermann*, Julian Frick* and Markus Paulmichl. University of Salzburg, Institute of Zoology, A-5020 Salzburg

Gossypol, a polyphenolic substance from cotton seeds induces both, antifertile and general toxic effects. We examined the influence ot this substance on the plasma membrane ion conductances of Madin Darby canine kidney (MDCK) cells by using intracellular recordings, the patch clamp technique and calcium imaging on a confocal laserscanning microscope. Gossypol in a concentration of 10µM causes a fast and reversible depolarization of the membrane potential from a resting value of -49,7 ± 1,1 mV to -20,3 ± 3,3 mV, accompanied by a decrease of the membrane resistance. The K_D-value is 8µM. The gossypol induced depolarization is completely blocked by 100µM amiloride, but was not blocked by up to 1µM tetrodotoxin (TTX). In patch-clamp experiments it was found that this substance activates directly amiloride sensitve sodium channels. Measurements with the calcium-indicator Fluo-3 show that the free intracellular calcium was not changed in the presence of gossypol. Our results indicate that gossypol activates a voltage- and time independent sodium channel in MDCK cells.

5.8

CYCLIC AMP DOES NOT ACTIVATE Ca^{2+}-BLOCKABLE POORLY-SELECTIVE CATION CHANNELS IN THE APICAL MEMBRANE OF A6 CELLS. W. Van Driessche, P. De Smet* and J. Simaels.* Laboratory for Physiology, KULeuven, 3000 Leuven (Belgium).

Transepithelial currents and noise were recorded to investigate Ca^{2+}-blockable cation-selective channels in A6 epithelia. Tissues were exposed to Ca^{2+} free K_2SO_4 Ringer solutions on the mucosal side and Ca^{2+} containing Na_2SO_4 on the serosal side. Noise analysis revealed the presence of a Lorentzian component in the power density spectrum of the fluctuation in current. Inward oriented currents carried by K^+ as well as the Lorentzian component were completely blocked by mucosal Ca^{2+}. Previously [Faseb J., 6, A1195, 1992] we reported that the cation-selective pathway is markedly activated by reducing the tonicity of the serosal solution suggesting the involvement of cell volume in the regulatory mechanism. The present study deals with the possible role of cAMP in the activation process of the cation-selective pathway. The major problem in this study is that cell volume is also altered by increases in Cl^- permeability of the apical membranes following the action of cAMP. We therefore incubated the epithelia with Cl^- free SO_4^{2-} solutions on both sides. Under these conditions the cation-selective pathway is only transiently activated by hypotonicity. However addition of SITS (1 mM) to the serosal bath caused a steady activated permeability. Using these conditions as control we studied the effect of serosal oxytocin (0.1U/ml), forskolin (10 µM) and cAMP (1 mM). All these agents were unable to alter the Ca^{2+}-blockable Lorentzian noise and currents. We conclude therefore that the regulation of the permeability of the cation-selective pathway occurs independently from cAMP mediated mechanisms.

5.9

INTERNAL CALCIUM INCREASES THE ACTIVITY OF CYCLIC-AMP DEPENDENT SODIUM CHANNELS IN *Pleurobranchaea*. L.C. Sudlow* & R. Gillette*. (SPON: P.M. Best). U. of Ill., Urbana Il 61801.

Cyclic-AMP-activated Na^+ currents ($I_{Na,cAMP}$) underly neuromodulator induced bursting in *P. californica* neurons (Green & Gillette, 1988, J.Neurophys. 59:248). We have characterized the interactions of cAMP and $Ca^{++}{}_i$ on $I_{Na,cAMP}$ using inside-out patches from serotonergic pedal neurons of *P. californica*. An inward current of -2.08 ± 0.19 pA (µ ± sem, n=8) with a 22 pS conductance was activated by 1mM cAMP. As the saline contained no ATP, phosphorylation does not appear to be required for activity. In 1mM cAMP, two subtypes of channels were activated. Type 1 had a percent open time (Po) of 2.84 ± 0.45% (n=8) and mean open time (MOT) was 2.46 ± 0.46 msec. Type 2 had a Po of 63 ± 1.92% (n=3) and a MOT of 47.04 ± 10.73 msec. The I-V relationship demonstrated a decrease in current at hyperpolarizing potentials and an increase at depolarizing potentials. When $Ca^{++}{}_i$ and cAMP were applied type one Po increased in a $[Ca^{++}]_i$-dependent manner but there was no change in the amplitude of the openings. Po (13.9%) and MOT (3.57 msec) were maximal in 200 nM $Ca^{++}{}_i$ with cAMP. $Ca^{++}{}_i$ entering during bursting augments the activation of $I_{Na,cAMP}$ by cAMP. From the I-V curve, depolarization causes an elevation in $I_{Na,cAMP}$. This positive feedback contributes to the excitation during bursting. This research was supported by NIH RO1 NS26838 and PHS 5 T32 MN18412.

5.10

CELL MEMBRANE POTENTIAL OSCILLATIONS IN NIH 3T3 FIBROBLASTS EXPRESSING THE HA-RAS ONCOGENE.

E. Wöll*, S. Waldegger*, F. Friedrich*, M. Ritter* and F. Lang* (SPON: M. Paulmichl) Inst. for Physiology, Univ. of Innsbruck, Austria

To elucidate the participation of ion channel activation in the cellular cascade following activation of ras oncogene, experiments were performed in NIH 3T3 fibroblasts expressing the ras oncogene (+ras) and fibroblasts not expressing the oncogene (-ras). In -ras and +ras bradykinin leads to a single transient hyperpolarization of the cell membrane. In +ras, however, this hyperpolarization is followed by permanent oscillations of cell membrane potential due to intermittent activation of Ca^{2+} sensitive, inwardly rectifying K^+ channels (~60 pS). Fura II measurements indeed show bradykinin induced oscillations of intracellular calcium activity. The oscillations are abolished in the absence of extracellular Ca^{2+}, in the presence of cadmium, lanthanum or nifedipine, but not after addition of diltiazem or verapamil. The frequency of the oscillations is decreased upon reduction of extracellular calcium from 1.2 to 0.2 mM. On the other hand the oscillations are blocked by TMB-8, a substance known to block intracellular Ca^{2+} release. Furthermore oscillations of cell membrane potential were observed in some cells after stimulation of Ca^{2+} entry by ionomycin. The growth of +ras in serum depleted media is significantly blunted in the presence of nifedipine, whereas the cell number of -ras cells is not significantly altered in the presence of the drug. In conclusion, expression of ras oncogene leads to periodic release of intracellular Ca^{2+} triggered by bradykinin induced influx of extracellular Ca^{2+}. These events are apparently required for the stimulation of cell proliferation in ras oncogene expressing cells.

5.11

DIALYSIS OF LACTOTROPHES WITH ANTISENSE OLIGONUCLEOTIDES ASSIGNS G-PROTEIN SUBTYPES TO THEIR CHANNEL EFFECTORS. Alex J. Baertschi, Yves Audigier*, Pierre-Marie Lledo*, Jean-Marc Israel*, Joel Bockaert* and Jean-Didier Vincent*. University of Virginia, Charlottesville, VA 22908 and INSERM/CNRS, France.

Sequential patch-clamping was applied to BSA gradient-enriched cultured lactotrophes from lactating rats, first to dialyze antisense oligodeoxyribonucleotides (AS) directed against G α protein mRNAs, and 48h later to record ion-current responses to the prolactin-release inhibitor, dopamine. The effectiveness and specificity of action of 6 types of AS were determined by their effects on the in vitro translation of αo, αi1, αi2, αi3, and αs. A total of 59 out of 240 cells could be investigated using the sequential patch clamp procedure in the absence of antibiotics. The typical decrease of the voltage-activated calcium-current in response to 10 nM dopamine was diminished or abolished by AS, in correlation with the inhibition of in vitro translation of the αo subunit. The typical increase of the voltage-activated potassium-current in response to dopamine was abolished by AS directed against αi3 but not αo mRNA. Control experiments showed that culture conditions or loss of receptor affinity for dopamine were not responsible for the loss of response. The results suggest that dopamine D2 receptors are linked via αo to calcium-channels, and via αi3 to potassium-channels. This approach may be applied to any cultured cell to study the roles of specific members of a protein family.

5.12

IMMUNOLOGICAL ISOLATION OF A POTASSIUM CHANNEL PROTEIN FROM BASOLATERAL MEMBRANES OF NECTURUS ENTEROCYTES. William P. Dubinsky*, Otilia Mayorga-Wark* and Stanley G. Schultz. Department of Physiology & Cell Biology, University of Texas Medical School, Houston, TX 77225.

We have recently demonstrated that a peptide composed of the N-terminal 22 amino acids of the *Drosophila* Shaker B K^+ channel protein, that is responsible for the inactivation of this A-type channel, blocks a voltage-gated K^+ channel present in the basolateral membrane of *Necturus maculosa* small intestinal enterocytes (Proc. Natl. Acad. Sci., 89:1770-1774, 1992). We now report that antibodies to this "inactivating" peptide interact with a 140 kDa protein in solubilized basolateral membranes from *Necturus* enterocytes. Asolectin vesicles reconstituted with the full complement of solubilized basolateral membrane proteins display Ba-inhibitable ^{86}Rb uptake that is abolished by immunoprecipitation of this protein. Asolectin vesicles reconstituted with this protein eluted from an antibody-affinity column display ^{86}Rb uptake that is abolished by boiling the eluate. Finally, incorporation of this protein into planar phospholipid bilayers reconstitutes single channel activity. We conclude that a 140 kDa protein present in basolateral membranes of *Necturus* enterocytes possesses K^+ channel activity and that this protein is antigenically similar to the type-A K^+ channel present in the flight muscles of *Drosophila melanogaster* encoded by the Shaker B locus.

5.13

THE PRIMARY STRUCTURE AND FUNCTIONAL CHARACTERIZATION OF A MAMMALIAN ATP-SENSITIVE POTASSIUM CHANNEL CDNA. K. Ho*, P.M. Vassilev*, M.V. Kanazirska*, J. Lytton* and S.C. Hebert. Brigham & Women's Hospital, Harvard Medical School, Boston, MA 02115.

Evidence suggests that ATP-sensitive K^+ channels (K_{ATP}) are involved in such diverse physiological processes as pancreatic β-cell insulin secretion, regulation of vascular smooth muscle tone, ischemia-induced changes in cardiac myocyte electrical activity, and K^+ secretion by the nephron. We report the isolation of a K_{ATP} channel cDNA, ROMK1, by expression cloning from a rat kidney outer medulla library. Using patch clamp techniques, K^+ currents in excised inside-out patches from ROMK1 mRNA-injected *Xenopus* oocytes exhibited properties of K_{ATP} channels: inward rectification in symmetrical K^+ solutions, single channel conductance of 44 pS, high open probability, slight voltage-dependence (P_o = 0.40, 0.71 at Vm = -90, -30 mV, respectively), high K^+ selectivity (P_{Na}/P_K = 0.025), and channel rundown. Channel activity could be maintained by excision of patches into bath solution containing 25 μM MgATP. However, application of 2.5 mM ATP to the cytosolic face of these patches resulted in reversible inhibition of channel activity. The cDNA sequence predicts a protein of 391 amino acids (M_r = 43K) consistent with the product obtained by *in vitro* translation of ROMK1 mRNA. Database comparison revealed no significant similarities. Hydropathy analysis suggests a strikingly novel topology that departs from the basic structural design characteristic of the voltage-gated and second messenger-gated ion channel superfamily. Only two membrane-spanning segments (M1, M2) flank an H5-like region; homology in this latter region is greatest in a P segment (59%) and suggests a common ancestry with known K^+ channels. A typical S4 segment is absent, although a region (M0) preceding M1 displays limited homology with K^+ channel S4 sequences (Shaker A, 36%). Following the M2 segment is a single putative ATP-binding site identified by a Walker type A motif. In sum, ROMK1 mRNA is sufficient for expression of a functional K_{ATP} channel in oocytes. The novel topology of the predicted protein appears to define a new class of ion channel proteins. Supported by NIH DK37605 & DK08487.

5.14

EXPRESSION OF A UNIQUE RENAL EPITHELIAL K CHANNEL IN *XENOPUS* OOCYTES Paul A. Welling* and Gerhard Giebisch.
Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven CT 06510

Potassium homeostasis and urinary potassium excretion are critically dependent on a small conductance, voltage-independent, inwardly-rectifying K channel, Kas, that is expressed in the renal cortical collecting duct. Based on the biophysical properties of Kas and the observation that the channel is inhibited by both ATP and sulfonylurea agents, it is likely that Kas is related to a family of ATP-sensitive K channels whose primary structures remain undetermined. Accordingly, to begin to elucidate the molecular basis of this channel, an expression cloning strategy has been initiated. Poly A⁺ RNA was extracted from confluent monolayers of cultured M1-CCD cells and injected into Dumont stage V-VI, defolliculated *Xenopus* oocytes. Potassium-channel activity was assayed by examining barium-sensitive (0.5mM $BaCl_2$) currents ($I_{K(Ba)}$) with the two-microelectrode voltage-clamp technique. 3-6 days following injection, RNA-injected oocytes developed $I_{K(Ba)}$ of -312 ± 37 nA at -90 mV in 50 mM K. In contrast $I_{K(Ba)}$ was not detected in sham-injected oocytes. Substitution of K with Na shifted the RNA-dependent, barium-sensitive reversal potential by 51mV/decade change in K. However, the newly expressed K channels sustained Rb currents with a inward-slope conductance of 1:4 relative to K. In the presence of 50mM K, the newly-expressed K currents exhibited inward-rectification with an inward to outward slope conductance ratio of 3:1. Consistent with Kas expression, tolbutamide (1mM) or glibenclamide (500 μM) but not TEA (25mM) completely inhibited $I_{K(Ba)}$. K channel expression was enhanced over 3 fold by 2.0-2.5 Kb and 3.5-4.5 Kb sized-fractionated M1-CCD Poly A⁺ mRNA. Both fractions exhibited identical biophysical and pharmacological properties. However, the K channel induced by size-fractionated mRNA lost sensitivity to the sulfonylurea agents. In summary: 1) a barium sensitive pathway induced by CCD mRNA exhibits biophysical and pharmacological characteristics in common with the secretory K channel of the renal cortical collecting duct, 2) the size fractionation studies suggest that the conductive K pore may be biochemically distinct from the sulfonylurea receptor.

5.15

A DIFFERENTIATION-RELATED AND VOLTAGE-GATED K^+ CURRENT IN ML-1 CELLS . L. LU. Wright State University, Dayton, OH 45431

A voltage-gated K^+ current in ML-1 human myeloblastic leukemia cells is related to the cell differentiation. In immature, proliferating ML-1 cells, the K^+ current can be activated by depolarization of the membrane potential to above -60 mV. Inactivation of the current is voltage-dependent and follows a simple exponential time course with a time constant (τ_i) of 900 ms at 0 mV. The current is inhibited by 4-aminopyridine (IC_{50} of 80 μM at 0 mV), but is much less sensitive to tetraethylammonium (TEA) or Ba^{2+}. In the early stages (at 7 hours) of differentiation induced by addition of 0.5 nM TPA in tissue culture, the current demonstrates suppressed activation and accelerated inactivation (10-fold decrease in τ_i). At later stages of differentiation (3 days), the current is essentially completely suppressed. We also observed that dramatic alterations in the K^+ current when TPA was added in the whole-cell patched cells. Amplitude of the voltage-activated current is initially increased within 4 min after introduction of 10 nM TPA in the bath solution and later suppressed at approximately 30-50 min. These findings indicate that the voltage-gated K^+ current in ML-1 cells can be regulated by TPA and is dramatically altered early in TPA-induced differentiation by the modulation of channel proteins (changes in the current activation and inactivation), and later by completely suppression of the current .

5.16

DISTINCT POTASSIUM EFFLUX PATHWAYS IN THE APICAL MEMBRANE OF GUINEA PIG GALLBLADDER EPITHELIAL CELLS. P. J. Gunter-Smith and K. D. Sample,*, Dept. of Physiol., AFRRI, Bethesda, MD 20889.

We used ^{86}Rb fluxes and potassium channel blockers to evaluate K efflux pathways in guinea pig gallbladder epithelial cells. Previously, we demonstrated that Prostaglandin E_2 (PGE)-stimulated K efflux was blocked by TEA, but not by Charybdotoxin (CHTX). This suggested that the TEA- and CHTX-inhibitable, voltage-dependent apical K conductance, which is also activated by Ca^{2+} ionophore (Gunter-Smith, 1988), contributes little to PGE-stimulated K secretion. PGE-stimulated K efflux is also not blocked by apamine (10^{-8} M) making it unlikely that a Ca^{2+}-activated K channel underlies PGE-stimulated K secretion. PGE's stimulation of electrolyte secretion occurs primarily through cAMP-dependent pathways. However, in many cells, secretion is also elicited via Ca^{2+}-dependent processes. We, therefore, evaluated Ca^{2+}-dependent K efflux pathways by determining the effects of various K channel blockers on ionomycin (IONO)-stimulated ^{86}Rb fluxes. In the absence of blockers, 10^{-6} M IONO increased serosal to mucosal ^{86}Rb flux (J_{sm}) from 0.33± 0.10 to 0.73±0.21 μEq/cm²·h. There was no effect on the mucosal to serosal flux (J_{ms}) increasing J_{net} from -0.17±0.08 to -0.46±0.19 μEq/cm²·h. In contrast to our results with PGE, this increase in J_{sm} was blocked by both 10^{-2} M TEA and 10^{-8} M CHTX (J_{sm} = 0.24±0.05 and 0.21± 0.04 μEq/cm²·h, respectively). These results suggest that K secretion can occur through at least two separate Rb permeable pathways, which are activated by different intracellular second messengers.

5.17

ANION DEPENDENCE OF BASOLATERAL K+ CHANNELS IN HT-29/B6 MONOLAYERS. Beate Illek, Horst Fischer, Klaus-M. Kreusel, Ulrich Hegel, and Wolfgang Clauss (Spon: Terry E. Machen). Dept. of Vet. Physiol., and Dept. of Clin. Physiol., Free University Berlin, FRG.

The effect of the anions Cl^-, Br^-, NO_3^- and gluconate on basolateral K^+ channels was investigated in the Cl^- secretory colon carcinoma cell clone HT-29/B6. Cells grown as polarized monolayers on permeable supports were mounted in Ussing chambers. The apical membrane was permeabilized with amphotericin B (10 µM) and a mucosal-to-serosal K^+ gradient (150:5) was applied as a driving force. This maneuver activated a volume-sensitive K^+ channel due to cell swelling when the external Cl^- concentration (Cl_o) was stepwise increased. Transepithelial K^+ current (I_K) and current noise were measured. In symmetrical solutions of the small anions Cl^-, Br^- and NO_3^- a significant I_K was induced (500±16.8 µA/cm², n=40; 530±141 µA/cm², n=3; and 152±18.1 µA/cm², n=6; respectively), however not with gluconate as the principle anion (I_K=17.9±2.6 µA/cm², n=10) which cannot penetrate the amphotericin pores. This indicates that the I_K required a permeable anion in the sequence $Br^- \geq Cl^- > NO_3^-$. Corresponding transepithelial resistances significantly dropped with Cl^-, Br^- and NO_3^-, but not with gluconate. The Rb^+ selectivity of the I_K (4:1) was unchanged in either Cl^- or NO_3^- containing solutions suggesting the presence of one K^+ channel type. A spontaneous Lorentzian component emerged in the noise spectra after permeabilization in the presence of Cl^-, Br^- and NO_3^-, but not with gluconate. The corner frequency (f_c) was the same with Cl^- or Br^- (f_c=29.8±1.6 Hz, n=31 and 23.0±3.0 Hz, n=5), however, with NO_3^- it was significantly reduced to 8.1±0.5 Hz (n=13, p<0.001). Neither addition of sucrose (cell shrinkage) nor reducing Cl_o from 148 to 74 mM affected f_c, indicating that NO_3^- directly acts on K^+ channel gating. We conclude that basolateral, swelling-*activated* K^+ channels are not only dependent on cell volume, but also are affected by the present anion. (Supported by DFG)

5.18

REGULATION OF CA^{++}-ACTIVATED K^+ CHANNELS IN THE NON-PREGNANT HUMAN MYOMETRIUM. W.Mahnert, W.Schreibmayer*, H.A.Tritthart* and N.E.Adelwöhrer*. Institute of Medical Physics and Biophysics, University of Graz, A-8010 Graz, Austria and Dept. of Gynecology and Obstetrics, University of Graz, A-8036 Graz, Austria.

Small parts of tissue were isolated from the fundus and the corpus of vaginally dissected uteri. A limited operation collective was studied (age of women 35-50 years, preclimacteric, not pathological) to get reproducible results. Strips of 1 cm length were cutted and contraction was isometrically measured. After 60 minutes of equilibration at 37 °C and under resting tension of 10 mN, spontaneous activity occured and experiments had been started. Single cells were isolated by enzymatic disaggregation with papain and collagenase. Electrophysiological experiments were performed using the patch-clamp technique in the cell-attached and excised inside-out configurations.

In almost every patch-clamp experiment with dissociated human myometrial cells Ca^{++}-activated K^+ channels (BK_{Ca}) were present. The open probability (P_o) of these channels was controlled by both the intracellular calcium concentration and the membrane potential. The channels of human myometrium had a conductance of 158 pS ± 5 between -20 and 20 mV of holding potential in $[K^+]_o/[K^+]_i$ of 5.4/140 mmol/l, resembling the large contuctances of big conductance calcium-activated channels in smooth muscle. Adenosine triphosphate (0.5 mmol/l) facing the cytoplasmic side of the patches (at 40 mV depolarization and pCa of 6) did not exert inhibition. Both hexoprenaline (mmol/l) and calcitonin gene-related peptide (CGRP, 0.1 mmol/l) increased the P_o of BK_{Ca}. Forskolin (10 mmol/l) as well as 8-bromoadenosine 3':5'-cyclic monophosphate (mmol/l) failed to activate the channel. Under isometric conditions the spontaneous activity of human myometrial strips was supressed by hexoprenaline (10 mmol/l) and CGRP (0.1 mmol/l). These effects were antagonized by 2 mmol/l tetraethylammoniumchloride (TEA). Blockade of phasic contractures by forskoline was antagonized by 3 mmol/l glibenclamide but not by TEA. Our results indicate that in the human non-pregnant myometrium activation of BK_{Ca} by hexoprenaline and CGRP is not mediated by adenylate cyclase pathway.

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5.19

WAY 120,491 ACTIVATES ATP-SENSITIVE POTASSIUM CHANNELS IN RAT TAIL ARTERY.

Philip B. Furspan, Univ. of Michigan, Ann Arbor, MI 48109

WAY 120,491 lowers blood pressure in a number of animal models. Its mechanism of action appears to be the selective opening of potassium channels in vascular smooth muscle cells. The patch clamp technique was to used to determine the specific potassium channel affected by WAY 120,491 in isolated tail artery cells of the rat. In the inside-out configuration, WAY 120,491 caused a dose dependent partial reversal of the ATP-induced inactivation of channel activity. Glyburide (10 µM) almost completely inhibited the effect of WAY 120,491. WAY 120,491 had no effect on Ca-activated K+ channels inactivated by the absence of calcium. In the whole cell configuration, 10 µM WAY 120,491 increased outward potassium current. 10 µM glyburide completely reversed the increase in current. WAY 120,491 appears to be a relatively specific activator of ATP-sensitive potassium channels in vascular smooth muscle.

5.20

ACTIVATION OF A DELAYED RECTIFIER IN CANINE JEJUNAL CIRCULAR SMOOTH MUSCLE BY FENAMATES. G. Farrugia, J.L. Rae and J.H. Szurszewski. Mayo Foundation, Rochester, MN 55905.

Jejunal circular smooth muscle cells (N=96) had a potassium selective delayed rectifier that was weakly holding voltage dependant, increased its open probability at -55mV and was fully open at about 5mV. In single cells, under whole cell voltage clamp using a perforated patch clamp technique, this current was the major contributor to the resting voltage. It was sensitive to quinidine block with 0.1µM blocking 50% of the current. Fenamates, such as flufenamic, mefenamic and niflumic acid, used as non-selective cation channel blockers in rat pancreas, were potent activators of this current, with no effect on other channels present. Activation was dose dependant with initial activation at about 75µM and with 1mM flufenamic acid increasing the current 500±350% (SD) from control (N=18). Activation of the current resulted in a marked shift in the resting voltage (mean =-22mV). Muscle cells with a less negative resting voltage showed the largest shift. The fenamate activated current reversed near E_k, suggesting a specific effect on potassium conductance. The effects were rapid (seconds) and reversible. Long term (minutes) application of high dose flufenamic acid prolonged the deactivation time, with the increase in current not fully reversible. The data suggest that a delayed rectifier is a major contributor to the resting voltage in jejunal circular smooth muscle and that fenamates cause hyperpolarization by activating this potassium current.

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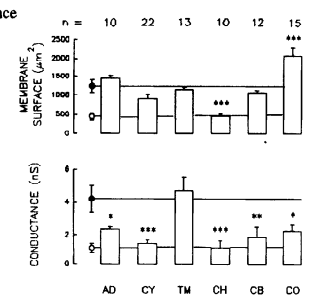
5.21

SYNTHESIS OF PLASMA MEMBRANE AND POTASSIUM CHANNELS IN EPITHELIAL CELLS (MDCK) Arturo Ponce* and Marcelino Cereijido. Dept. of Physiol. CINVESTAV, Apartado Postal 14-740, México, D.F.

Mature MDCK cells have a K conductance of 10.3 ± 2.1 nS (n=20), as determined with the whole cell clamp technique. Harvesting makes them lose up to 70% of their plasma membrane and 80% of their K conductance, presumably due to cleavage and removal of K channels. Upon replating cells restore surface and channels in a few hours. The use of inhibitors of mRNA (AD) and protein synthesis (CY), glycosilation (TM), exocytic fusion (CH), and agents that disrupt microtubules (CB) and microfilaments (CO) on the recovery of plasma membrane (fig above) and K channels (fig below) indicates that restoration of plasma membrane requires neither the synthesis of mRNA nor proteins, but does depend on exocytic fusion. On the other hand, restoration of channels, depends on the synthesis of mRNA and proteins as well as on exocytic fusion. Glycosylation is not necessary to restore membrane surface nor K channels. Reinsertion of channels also requires the participation of microtubules and microfilaments. **Legend of the Figure:** values found in control cells 30-45 min (o) and 4-6 hrs after plating (•). AD: Actynomicin D; CY: Cycloheximide; TM: Tunicamicin; CH: Chloroquine; CB: Cytochalasin B; CO: Colchicine.



5.22

REDOX ACTIVE AGENTS ALTER PULMONARY VASCULAR TONE. E.K. Weir, D.A. Peterson and S.L. Archer. VA Medical Center & University of Minnesota, Minneapolis, MN 55417

Hypoxia inhibits a K^+ current in pulmonary vascular smooth muscle (AJP 262:C882, 1992) causing membrane depolarization and calcium influx. We have suggested that redox status modulated by oxygen tension may alter pulmonary vascular tone (Herz 11:127, 1986). Duraquinone enhances electron transfer. It accelerates cytochrome C reduction by ferrous iron in vitro. Duraquinone (40 µM) increased cytochrome C reduction to 49±1% at 30 seconds, compared to 36±3% control (p<0.01). Duraquinone (100 µM) caused contraction of 7 endothelium-intact rat pulmonary artery rings (+412±43mg) and 7 endothelium-denuded rings (+297±41mg). Diamide is a thiol oxidant which is known to inhibit hypoxic pulmonary hypertension (Proc Soc Exp Biol Med 173:96, 1983). Diamide (400 µM) inhibited cytochrome C reduction in vitro. The reduction was 34±4% at 2 minutes, compared to 93±8% control. Diamide (100 µM) reversed the contractions caused by duraquinone (-430±67mg intact rings; -338±28mg denuded rings). The observation that an agent which enhances electron transfer, constricts, while an agent which inhibits electron transfer, relaxes pulmonary artery rings supports the hypothesis that changes in redox status can alter pulmonary vascular tone. This might occur by gating of a K^+ channel.

5.23

PHOSPHATE DEPLETION INHIBITES NH_4^+ PERMEABILITY IN OK CELLS
J.G. Chen, S.A. Kempson. Department of Physiology, Indiana University School of Medicine, Indianapolis, IN 46202.
Previous studies have shown that phosphate depletion (PD) causes impaired reabsorption of HCO_3^- in proximal tubule cell. The reabsorption of "new" bicarbonate is largely associated with the excretion of NH_4^+. To examine NH_4^+ permeability after PD, OK cells were Pi depleted by 3.5 hours in Pi free media. Intracellular pH was monitored by BCECF. Perifusion with 20 mM NH_4Cl leads to rapid alkalinization followed by acidification. We take the initial slope of acidification as index of NH_4^+ permeability across cell membrane. DIDS did not attenuate this response, making $Cl^- - HCO_3^-$ exchange an unlikely mediator of this acidification. NH_4^+ permeability was reduced in PD cells by 34%: control=3.14±0.45, PD=2.06±0.56 mM NH_4^+/min, N= 7, P<0.005. $BaCl_2$, not amiloride, can abolish NH_4^+-induced acidification. Intracellular K^+ concentration decreased slower in PD than control after incubation with ouabain (0.1 mM) for 50 min. Final K^+ concentration was 0.23±0.02 (control), 0.28±0.01 mmol/mg protein (PD), P<0.01. Conclusion: NH_4^+ permeability is inhibited in PD OK cells. Mechanism may involve decreased K^+ conductance thereby reducing NH_4^+ transport through K^+ channels.

5.24

PLANAR BILAYER STUDIES OF K^+ AND Cl^- SELECTIVE ION CHANNELS IN SOYBEAN PHOSPHATIDE EXTRACT.
V. Ruiz-Velasco and L. J. Hymel. Tulane University School of Medicine, New Orleans, LA 70112

We have observed distinct ion channel activities in approximately 90% of planar bilayers formed from vesicles consisting of a mixture of acetone-washed soybean phosphatide extract (SBL) plus cholesterol (6/1 ratio by weight). SBL contains proteolipids which may be the source of channel activity. The channels are spontaneously active between ± 150 mV. They appear singly or in small numbers and are distinguished by their long open times of seconds to minutes. A rapid flickering mode with open times in the msec range was sometimes observed. Open channel states are individually very distinct, but collectively range from 3 to 10 pS and sometimes higher in 100 mM KCl. Smaller subconductance states are frequently observed. Current-voltage relations measured in 350-100 mM KCl gradients demonstrated that the channels are of two types, one K^+ selective (E_{rev} = -23mV, P_K/P_{Cl} = 6.7, g_{slope} = 3 pS) and one weakly Cl^- selective (E_{rev} = +9mV, P_{Cl}/P_K=1.9, g_{slope} = 10 pS). The K^+ channel can be blocked by 50 mM Ca^{2+}. These activities constitute a new class of easily studied, molecularly simple ion channels whose presence must be considered when using SBL for reconstitution studies of membrane transport proteins. Supported by the Louisiana Board of Regents (LEQSF(1991-94)-RD-A-34).

5.25

STUDY OF CONDUCTANCE PROPERTIES OF LIVER MICROSOMAL MEMBRANES AFTER FUSION WITH AN ARTIFICIAL BI-LAYER MEMBRANE.
Ashutosh Tripathy and H. Ti Tien.* Dept. of Physiology, Michigan State University, E. Lansing, MI 48824.

Liver microsomes have been fused with a BLM (bi-layer membrane) with an aim to reveal the inositol trisphosphate gated ion channels in the liver ER (endoplasmic reticulum) membrane. Fusion was achieved by using an asymmetric choline chloride buffer solutions. In some cases, we have successfully used potassium chloride or potassium acetate as the osmoticant. In cis 250mM, and trans 50mM choline chloride solution, the reversal potential is about 25mV. In cis 200mM, and trans 0 mM potassium chloride or potassium acetate, the reversal potential is about 80 - 100mV. Both cases demonstrate appreciable chloride conductance of the BLM after microsomes have fused. Fusion occurred in steps and the membrane conductance increased from 5 to 200 - 400pS. In some cases single potassium and chloride channels were observed. After microsomal fusion, both the cis and trans sides of the BLM were perfused with only calcium containing solutions to reveal inositol trisphosphate gated ion channels.

This work was supported by NIH.

5.26

RAT CHIP28 WATER CHANNEL GENE IS HIGHLY EXPRESSED IN RENAL CORTEX AND MEDULLA.
Peter M.T. Deen*, Bé Wieringa* and Carel H. van Os. Univ. of Nijmegen, 6500 HD Nijmegen, The Netherlands.

High water permeability of proximal tubules and erythrocytes is due to membrane proteins serving specifically as water channels. The cDNA encoding the human red blood cell water channel CHIP28 has recently been isolated. Expression of CHIP28 in Xenopus oocytes proved that this protein is a water channel (Preston et al., Science **256**, 385, 1992). Radiation inactivation studies revealed the same size for the water channel in proximal tubules and in erythrocytes (van Hoek et al., BBRC **184**, 1331, 1992). Therefore, we attempted to isolate a cDNA clone for CHIP28 from rat kidney. First, by means of PCR a 600 bp product coding for part of the human CHIP28 was obtained from a fetal liver cDNA library. With this probe a rat kidney cDNA library was screened and with an additional reverse transcriptase PCR technique a full-length rat cDNA clone of 2.7 kb was isolated. The similarity with amino acids sequence of the human red blood cell CHIP28 is 93%. The protein encoding sequence is >80% identical. Subsequently, mRNA was isolated from the rat renal outer cortex and the inner medulla. By means of Northern blot hybridization it is shown that CHIP28 is well expressed in the cortex and the medulla. This contrasts with immunohistochemical studies showing that antibodies raised against human red blood cell CHIP28 only cross-reacted with the proximal tubule (Denker et al., JBC **263**, 15634, 1988). The level of CHIP28 expression was not different in diuresis and antidiuresis. In conclusion, the rat renal CHIP28 is 93% identical to human erytrocyte CHIP28. Northern blot analysis strongly suggests that water channel proteins in the proximal tubule and the collecting duct are identical.

5.27

MOLECULAR BIOLOGY OF CHIP28 WATER CHANNELS.
Peter Agre*, Gregory M. Preston*, Barbara L. Smith*, Jin Sup Jung*, Chulso Moon*, Tiziana P. Carroll*, and William B. Guggino. Johns Hopkins Univ School of Medicine, Baltimore, MD 21205

Transmembrane water movement in red cells and renal tubules thought to be mediated by water-selective channels. CHIP28 is an integral protein which comprises nearly 4 % of apical brush border membrane protein in renal cortex. Native CHIP28 proteins apparently contain three nonglycosylated subunits and one N-glycosylated subunit. The 2.9 kb cDNA for red cell CHIP28 was isolated and encodes a 269 amino acid polypeptide. The proposed membrane topology indicates that CHIP28 protein has 6 bilayer spanning domains. Moreover amino acid sequences of the N- and C-terminal halves are distantly related but are apparently oriented 180° to each other within the membrane. CHIP28 belongs to a family of integral membrane proteins with incompletely defined functions including MIP26 (bovine lens), and a series of plant proteins including TIP (lines water containing tonoplasts) and TUR (a root protein induced by water deprivation). CHIP28 protein was studied for a potential role in water channel activity. *Xenopus* oocytes injected with in vitro transcribed CHIP28 mRNA exhibited strikingly increased osmotic water permeability (P_f = 220 μm/sec) with low Arrhenius activation energy (E_a < 4 kcal/mol), and was reversibly inhibited by $HgCl_2$. Two electrode voltage clamp analyses of CHIP28 oocytes failed to reveal an increase in ion conductance. Quantitation of CHIP28 expression by dilutional immunoblotting revealed that each CHIP28 monomer may permit up to 10^{-14} ml of water flow per second. These and other studies indicate that CHIP28 is the constitutive water channel of red cells and renal tubules.

5.28

COMPARTMENTALIZATION AND COVALENT MODIFICATIONS (ISOELECTRIC SPECIES) OF ANNEXINS IN HUMAN AMNION
W.V. Everson* and L. Myatt* (SPON M. McLaughlin). College of Medicine, University of Cincinnati, Cincinnati OH 45267.

We have previously shown that annexins I-VI are present in human amnion in both a calcium-dependent and a calcium-independent membrane-associated pool. The distribution of several annexins in these pools changes with labor and may be associated with increased phospholipase activity and production of prostaglandins. The basis for the differential membrane association of annexins has not been identified, although covalent modifications may alter their binding affinity. We developed a method using 1-D IEF-PAGE followed by immunoblotting to separate individual isoelectric species of annexins and determine the distribution in the calcium-dependent and calcium-independent pools. Annexin I resolved into three major species: the major basic species (pI 8.0) was predominately found in the calcium-independent pool, an acidic species (pI 7.0) preferentially distributed into the calcium-dependent pool, while the third form (pI 7.3) was distributed equally in both pools. Annexin II was present in 3 major species: The major acidic form (pI 6.9) and several minor forms clustered around pI 6.9 were found in the calcium-dependent pool while the basic forms (pI 7.6, 8.0) were preferentially distributed in the calcium-independent pool. The preferential distribution of individual isoelectric species of these two annexins into calcium-dependent and calcium-independent pools suggests either that covalent modifications have a causal relationship to the localization or that enzymes which covalently modify annexins are compartmentalized. Supported by NIH HD27603 (WVE) and HD26167 (LM).

5.29

ISOLATION AND RECONSTITUTION OF SINGLE CONNEXONS AND CONNEXON PAIRS. P.J. Donaldson, J. Bond* and J. Kistler*, Department of Cellular and Molecular Biology, University of Auckland, Auckland, New Zealand.

The fiber cells of the lens are extensively coupled by gap junction channels which contain the connexin related membrane polypeptide MP70 (Kistler et al, J.Struct.Biol. 103:204-211, 1990). Triton X-100 solubilised lens membrane protein preparations are enriched in MP70 and contain a mixture of connexons (hemi-channels) and connexon pairs. These preparations exhibit channel activity upon reconstitution into planar lipid bilayers (Donaldson & Kistler, J.Memb.Biol. in press). Two novel conductances of 45 and 90pS (in 150mM KCl) are observed both of which have a linear current-voltage relationship, are halothane sensitive (5mM) and lack cation selectivity (Na:K). The 45pS activity is symmetrically voltage dependent as would be expected for connexons pairs, while the 90pS activity exhibits an asymmetric voltage dependence consistent with a single connexon. These structure-function relationships are presently being tested by screening for suitable detergents and solubilization conditions to obtain either predominately connexons or connexon pairs. These structures are further separated by gradient centrifugation on the basis of their different sedimentation constants of 9 and 16 respectively, and subsequently analyzed for channel activity with the planar bilayer system.

5.30

GAP JUNCTION VOLTAGE, DEPENDENCE: WHAT SINGLE CHANNEL ANALYSIS REVEALS ABOUT THE MACROSCOPIC CONDUCTANCE. D.C. Spray, M. Chanson,* M.B. Rook* and A.P. Moreno*. A. Einstein Coll Med, Bronx, NY 10461

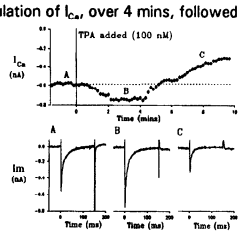
Gap junctions are sensitive to transjunctional voltage (V_j) such that junctional conductance (g_j) decreases monexponentially with V_0 values (V_j at which g_j is reduced by 50%) that are specific for the type of gap junction protein (connexin) expressed. It has been hypothesized that voltage dependence arises from decreased open probability (P_o) of the channels and that the minimal conductance (g_{min}, reached at high V_js) is due to nonzero P_o of the channels. Studies on several cell types have addressed these issues. In Schwann cells, voltage dependence of g_j is quite steep (V_0~20 mV) and is well fit by a Boltzmann distribution without a g_{min} component. Channels are of a single size population (unitary conductance, γ_j, ~40 pS), and P_o is fit by the same Boltzmann relation describing g_j. In SKHep1 cells (which express connexin40), and in SKHep1 cells transfected with *Xenopus* connexin38, voltage dependence is also steep, but a g_{min} of >10% is required for the Boltzmann fit. At low V_js, γ_j is ~30 and ~40 in these cells, but at high V_j, smaller channels are present. In SKHep1 cells transfected with human connexin43, voltage dependence of g_j is less steep (V_0~55 mV), and g_{min} is almost 50%. At low g_j values, γ_j is ~60 or ~90 pS, depending on phosphorylation state. At high V_j, a smaller, less voltage dependent channel becomes evident, accounting for g_{min}. Together, these data indicate that voltage dependence of g_j is attributable to decreased P_o and that for certain connexins a voltage independent substate exists that accounts for g_{min}

CALCIUM TRANSPORT AND REGULATION OF INTRACELLULAR CALCIUM

6.1

THE EFFECTS OF TPA ON I_{Ca} AND EXCITATION-CONTRACTION COUPLING IN RAT HEART CELLS CHANGES DURING DEVELOPMENT. M.S. Kirby*, A. Lokuta, S. Gea, C.F. Neubauer, T.B. Rogers & W.J. Lederer*, Univ. Maryland, Baltimore, MD 21201.

We have previously shown that the phorbol ester TPA, which activates protein kinase C (PKC), has no effect on Ca^{2+} current (I_{Ca}) in adult rat ventricular myocytes. The reduction in the Ca^{2+} transient and twitch produced by PKC activation may depend on an effect mediated by the sarcoplasmic reticulum (SR) or Na^+/Ca^{2+} exchange (Kirby et al., Biophys. J. 59, 281a). In contrast, TPA increased I_{Ca} (Dosemeci et al., Circ. Res., 62, 347-357), and first increased and then decreased Ca^{2+} channel open probability in patch-clamp studies (Lacerda et al., Nature, 335, 249-251), in cultured neonatal rat heart cells. The figure shows an experiment in a cultured neonatal rat heart cell where TPA was present for a prolonged period. Under these circumstances (and unlike the adult cells) there was an initial stimulation of I_{Ca}, over 4 mins, followed by a subsequent inhibition of I_{Ca}, suggesting that at least part of the effect of PKC activation in neonatal cells is via I_{Ca}. Cells were superfused with K^+ free Tyrode plus 2 mM Ca^{2+} and 10 μM TTX, at 35°C and pH 7.4 using standard techniques. The patch solution was (mM): 125 CsCl, 5 MgATP, 20 HEPES, 10 EGTA, 0.5 NaH_2PO_4, 7.5 NaCl, Indo 0.07, pH 7.15 and Ca^{2+} of 100 nM at 35°C. The cell was held in voltage clamp at -45 mV and stepped to 0 mV for 150 ms at 0.1 Hz. I_{Ca} was blocked by cadmium (100 μM).



The source of Ca^{2+} for contraction changes during development, and is predominantly from I_{Ca} in the neonate and from the SR in the adult. Our data suggests that PKC activation modifies excitation-contraction coupling at different sites, dependent on the age of the heart. (Funded by N.I.H. and A.H.A.-MD. Affil. Postdoc. Fellowship).

6.2

PROPOFOL AND MIDAZOLAM DECREASE CALCIUM CURRENT IN CANINE VENTRICULAR CELLS. V. Bérczi*, N. Buljubasic*, D.F. Supan*, J.P. Kampine, Z.J. Bosnjak. Department of Anesthesiology, Medical College of Wisconsin, WI 53226

Intravenous anesthetics are known to have negative inotropic effect on the heart, and alterations of transmembrane ionic fluxes by these agents may be one of the mechanisms of myocardial depression. The purpose of the present study was to investigate and compare the effects of 30 and 60 µM propofol and midazolam on L-type Ca^{2+} current (I_{Ca}) in single canine ventricular cells using whole-cell patch-clamp technique.

Single myocytes were isolated from the canine left ventricle using collagenase. Dispersed cells were placed in the microscopic chamber (22°C), with 10 mM $BaCl_2$ being used as a charge carrier. Intrapipette solution contained 130 mM $CsCl_2$. I_{Ca} was elicited by stepwise depolarizing pulses from a holding potential of -40 mV to +40 mV. Peak I_{Ca} decreased in the presence of propofol by 21.7±7.6, 32.5±4.5%, while midazolam produced depression of 30.4±3.1 and 55.3±6.3% (n=5-10; p<0.05 in each group), which was reversible upon washout. The direct depressant effect of intravenous anesthetics on the cardiac L-type I_{Ca} may be one of the mechanisms involved in the negative inotropic response produced by these agents.

6.3

VOLTAGE AND TIME-DEPENDENT POTENTIATION OF L-TYPE CALCIUM CURRENTS IN SKELETAL MUSCLE CELLS. A. Sculptoreanu, T. Scheuer and W. A. Catterall. (SPON: W. E. Crill) Dept. Pharmacology. Univ. of Washington. Seattle, WA 98195.

Activity-dependent facilitation of L-type Ca channels has been described in chromaffin cells and cardiomyocytes. In skeletal muscle cells, activation of Ca currents by depolarizing voltages has been shown to accelerate activation rates on subsequent stimulation. Here, we report large, voltage- and time-dependent potentiation of L-type Ca currents in rat skeletal muscle myoballs which requires phosphorylation of the channels by cAMP dependent protein kinase.

Ca currents were recorded in 20, 40 and 110 mM Ba solutions in whole-cell voltage clamp configuration. The time- and voltage-dependence of Ca channel potentiation was defined using a three pulse protocol. A 300 ms test pulse near the threshold of Ca current activation was followed by a 9 s period in which the voltage was returned to -96 mV; this was followed by a conditioning pulse to a positive voltage of variable duration and then a depolarizing test pulse identical to the first. Currents elicited by the third pulse in the sequence were potentiated in comparison to those in the first and lengthening conditioning pulse duration from 50 to 1000 ms increased that potentiation. Stronger conditioning depolarizations gave larger potentiations with faster onsets. Conditioning pulses longer than 1000 ms inhibited the potentiation. Voltage and time-dependent reversal of potentiation occurs upon hyperpolarization and has a half time near 40 ms at -60 mV. Potentiation of L-type Ca currents following depolarizing conditioning pulses was associated with a stable and reversible negative shift in the voltage dependence of activation. Inclusion of 100 nM-10 µM peptide inhibitor of cAMP dependent protein kinase largely inhibited the potentiation of Ca currents indicating that phosphorylation by cAMP-dependent protein kinase is required for these effects. This research was supported by NIH Grant NS22625. A. S. was a postdoctoral fellow of the Heart and Stroke Foundation of Canada.

6.4

SINGLE CHANNEL CONDUCTANCE AND SELECTIVITY OF PURIFIED SARCOPLASMIC RETICULUM CALCIUM RELEASE CHANNELS ISOLATED FROM NORMAL AND MALIGNANT HYPERTERMIA SUSCEPTIBLE PIGS Nirah H Shomer, James R. Mickelson*, and Charles F. Louis* (SPON: E. Gallant). Program in Veterinary Biology, University of Minnesota, St. Paul, MN 55108

We have previously demonstrated that the Arg^{615} to Cys^{615} mutation of the SR calcium release channel of malignant hyperthermia susceptible (MHS) pigs results in an abnormal regulation of single channel properties such that a three-fold higher concentration of cis Ca^{++} is required to inhibit the opening of MHS channels. To investigate whether the mutation affects the ion selectivity filter of the channel, we have examined whether there are abnormalities in the cation selectivity of this channel. Single Ca^{++} release channels isolated from MHS and normal pigs were incorporated into planar lipid bilayers in order to measure their monovalent cation conductances and their ion permeability ratios. Monovalent conductances in symmetrical 215mM XCl were: Li^+ 183±3 (n=21); Na^+ 474±6 (n=29); K^+ 771±7 (n=29); Rb^+ 502±10 (n=22); and Cs^+ 527±+5 (n=16). There was no significant difference between MHS and normal single-channel conductances for any of the monovalent cations tested. Permeability ratios for K^+ versus these five monovalent cations were measured under biionic conditions, and the channel was found to have the permeability sequence $Li^+ > Na^+ > K^+ \geq Rb^+ > Cs^+$, with no significant difference noted between MHS and normal channels. Ca^{++} regulation of channel gating is affected by the mutation, so the permeability ratio of Ca^{++} versus K^+ + was measured to test whether the ion selectivity for Ca^{++} was different in MHS and normal channels. $P_{Ca^{++}}:P_{K^+}$ was 18.5±4.0 and 14.2±3.2 for MHS and normal, respectively, and were not significantly different. We conclude that the Arg^{615} to Cys^{615} mutation does not affect the conductance or ion selectivity properties of the sarcoplasmic reticulum Ca^{++} release channel. Supported by NIH grant GM31382.

6.5

DOWN-REGULATION OF L-TYPE Ca^{2+} CHANNELS IN ISOLATED ARTERIAL MUSCLE BY PRETREATMENT WITH RECEPTOR AGONISTS. Paul H. Ratz, Adrienne Boothe, Frank A. Lattanzio, Jr., and Jim J. Sadighian. Eastern Virginia Medical School, Norfolk, VA 23501

In a previous study (Ratz and Lattanzio, Am J Physiol 1992; 262:C621-C627), pretreatment of rabbit femoral arterial strips with ser/thr protein phosphatase inhibitors reduced the ability of 110 mM KCl and 1 μM Bay k 8644, activators of L-type Ca^{2+} channels, to increase $[Ca^{2+}]_i$ and force compared to untreated tissues. The results suggest that increased phosphorylation may down-regulate L-type Ca^{2+} channels. The present study examined the proposal that similar down-regulation occurs, possibly as an autoregulatory mechanism, during activation by contractile receptor agonists. Tissues were contracted (pretreated) for 30 min with the α-adrenoceptor agonist, phenylephrine (PhE), then relaxed completely for 10 min and contracted with KCl. Force, $[Ca^{2+}]_i$, and the extent of myosin light chain phosphorylation produced at 5 min in pretreated tissues were about 50% weaker than responses produced in untreated tissues. Also, the degree of inhibitions of KCl-induced contractions were greater in tissues that were pretreated for longer durations and at higher [PhE]s and weaker in tissues relaxed for longer periods after the pretreatment. These data suggest that, in addition to generating cell messengers that increase force, PhE generates an autoinhibitory mediator with a short half-life that may reduce L-type Ca^{2+} channel activity.

6.6

FAST LIDOCAINE BLOCK OF SKELETAL AND CARDIAC MUSCLE SODIUM CHANNELS: DIRECT HYDROPHILIC AND HYDROPHOBIC ROUTES TO A COMMON SITE. Gerald W. Zamponi, Donald D. Doyle, and Robert J. French. University of Calgary, Alberta, Canada, T2N 4N1 & University of Chicago, Illinois, IL 60637.

We have investigated the block by lidocaine of single, batrachotoxin-activated sodium channels, from beef or sheep cardiac muscle, or from rat skeletal muscle using recordings from single channels incorporated into neutral planar phospholipid bilayers, bathed in symmetric 200mM NaCl, 20mM MOPS, pH 7.0. Lidocaine (0.3 - 9mM), applied to the extracellular or cytoplasmic sides, induced two kinetically distinct modes of block in cardiac channels. 'Fast' block appeared as a reduction in the apparent single channel current, and an increase in open-state noise, and was almost identical for cardiac and skeletal muscle channels. The 'slow' blocking mode was prominent only for cardiac channels, and appeared to be caused by binding of lidocaine to a closed state characteristic of the cardiac Na channel subtype. For fast block, unblocking rates were indistinguishable in magnitude or voltage dependence regardless of the side of application of the drug, suggesting that lidocaine reached the same site of action from either side of the membrane. In contrast, the blocking rate was enhanced by depolarization for internal application, but was voltage-independent for external application. When lidocaine was applied on one side of the membrane there was no significant accumulation on the other side; block was reduced to negligible levels by replacing the solution only on the side of drug application. Together, these observations indicate that lidocaine may reach the same site via two different pathways to cause the fast block. We suggest that: 1) from the intracellular side, the charged form of the drug approaches its receptor via a hydrophilic pathway, and 2) from the extracellular side, the neutral form of the drug approaches the same receptor directly via a hydrophobic pathway, but without completely crossing the membrane.

6.7

ANOTHER INTRANEURONAL CALCIUM CHANNEL?

Cécile Martin* and Richard Ashley* (SPON: Ian Hampton). Department of Biochemistry, University of Edinburgh, George Square, Edinburgh EH8 9XD, U.K.

Two distinct and specific channels, ryanodine-sensitive and inositol triphosphate-activated Ca^{2+} channels, are involved in intraneuronal Ca^{2+} regulation. Using the planar lipid bilayer technique to search for other intraneuronal Ca^{2+} channels, we have characterized another high conductance Ca^{2+} channel from rat brain endoplasmic reticulum membranes. Channel conductance in 50 mM $CaCl_2$ is 100 pS and the channel is permeable to Ba^{2+}, K^+, and Cs^+. It is, however, only poorly cation-selective (P_{Cs^+}:P_{Cl^-} = 4:1). The channel has been distinguished from other well-known intracellular and extracellular Ca^{2+} channels (it is insensitive to ryanodine, caffeine, heparin , verapamil and $InsP_3$), but it is difficult to assign it a specific cellular origin on the basis of reconstitution studies alone. The channel opens in bursts in a steeply voltage-dependent manner to allow maximum Ca^{2+} transport around zero mV with rapid inactivation nearer the resting potential of about -80 mV. This suggests that the channel may be sensitive to changes in the plasma membrane potential, and may even provide a direct physical link to the cell surface. In conclusion, we suggest that this channel may have a role in intracellular Ca^{2+} signalling and the operation of intracellular Ca^{2+} stores.

Supported by the Wellcome Trust.

6.8

$[Na^{2+}]_i$ AND $[Ca^{2+}]_i$ CHANGES RELATED TO AN INSULIN-SENSITIVE CATION CHANNEL. Julie E. M. McGeoch*, Anthony Morielli,* and Guido Guidotti. Harvard University, Cambridge, MA 02138.

Insulin causes a transient 38±14nM rise in $[Ca^{2+}]_i$ and a temporally related decrease in $[Na^+]_i$ in skeletal muscle (neonatal soleus) loaded with Fura-2,AM and SBFI,AM respectively. These changes were dependent on $[Ca^{2+}]_o$, inhibited by μ-conotoxin and not by TTX. A similar $[Ca^{2+}]_i$ transient was elicited by 8'-Bromo cGMP, confirming the involvement of a previously described insulin sensitive cation channel (McGeoch, J.E.M., and Guidotti, G. (1992) J. Biol. Chem. 267,832-841). These results demonstrate that Ca^{2+} can modulate $[Na^+]_i$. Via stimulation of a protein kinase, possibly Protein kinase C, insulin also reduces the Ca^{2+} release on muscle depolarisation. A 495±127nM $[Ca^{2+}]_i$ rise is decreased to 139±101nM when the muscle is pretreated with insulin prior to a depolarising dose of KCl. TPA gives the same effect (155±22nM). The kinase inhibitor HA1004, added prior to insulin blocks the insulin depression (436±149nM). HA1004 also blocks the reduction by TPA (795±361nM).

6.9

EFFECTS OF NH_4Cl ON SODIUM/PROTON (Na^+/H^+) EXCHANGE AND CYTOSOLIC CALCIUM IN MAN. **Peter Reusch*, Regina Reusch*, Winfried Siffert*, Johannes Mann*, and Friedrich C. Luft, Univ. Erlangen, 8520 Erlangen, FRG.**

To test the effect of metabolic acidosis on Na^+/H^+ exchange and to examine the relationship between Na^+/H^+ exchange and $[Ca^{2+}]_i$ in man, we measured both parameters in patients with a mean plasma creatinine of 8±2mg/dl (pH 7.34±0.06), in normal controls (pH 7.41±0.02), and in subjects before (pH 7.40± 0.01), and after (pH 7.26±0.04) NH_4Cl, 15 g for 5 days. Lymphocytes (L) and platelets (P) were loaded with BCECF and acidified with 10-50 mM/l propionic acid; $\delta pH_i/\delta t$ was determined at one minute. $[Ca^{2+}]_i$ was measured with Fura-2. The buffering capacity of the cells was not different in any experiments. Na^+/H^+ exchange was increased ($p<0.05$) in L and P of patients with renal insufficiency (L/P $\delta pH_i/\delta t$ at 50 mM: 0.079/0.083 vs. 0.49/0.68), although neither pH_i (6.95/7.05 vs. 7.01/7.07) nor $[Ca^{2+}]_i$ (72/118 vs. 75/122 nmol/l) were different from controls. NH_4Cl increased Na^+/H^+ exchange ($p<0.05$) in L ($\delta pH_i/\delta t$ at 50 mM: 0.085 vs. 0.049), but not in P (0.065 vs. 0.068) of normal subjects, compared to controls. However, NH_4Cl had no effect, either on pH_i (7.07/7.09 vs. 7.01/7.07), nor on $[Ca^{2+}]_i$ (74/116 vs. 75/122 nmol/l). NH_4Cl also caused a 2 kg weight loss, an increase in renin, aldosterone, UKV, UCIV, and UCaV (all $p<0.05$). We conclude that chronic, metabolic acidosis increases Na^+/H^+ exchange, but does not influence pH_i or $[Ca^{2+}]_i$ in L and P. Since, short-term metabolic acidosis influenced Na^+/H^+ only in L, but not in P, protein synthesis may be involved in increasing Na^+/H^+ exchange. Finally, these data do not support the notion that Na^+/H^+ exchange and $[Ca^{2+}]_i$ are closely and integrally related. (Sponsored by Siemens Erben A.G.)

6.10

CALCIUM AGONIST/ANTAGONIST ACTION ON SKELETAL MUSCLE HIGH ENERGY PHOSPHATE CONTENTS. **K. Sanchez* and E. T. Hays. Barry University, Miami, FL 33161.**

Both caffeine and theophylline at subcontracture levels (2mM) reduce high energy phosphate contents in isolated frog sartorius muscle. A variety of substances such as ryanodine, verapamil, nifedipine, and procaine are calcium agonists and/or antagonists. In many cases these substances can have both agonistic as well as antagonistic effects with one action at low concentrations and other actions at higher concentrations. Whereas low ryanodine (1-10 μM) has no effect on energy stores, higher ryanodine (100 μM) reduces energy stores and depletes high energy stores even further when combined with subcontracture levels of theophylline (2 mM). Low verapamil (0.1 mM) has no effect on energy stores and has little or no effect on theophylline-induced energy depletion. High verapamil (1 mM) alone reduces high energy stores while in combination with theophylline causes an even greater energy depletion. Procaine (1 mM) which blocks caffeine-induced depletion reduces but does not block completely theophylline-, ryanodine- nor verapamil-induced depletion. These results may suggest multiple sites for interactions of these agents.

(Supported by NIH-NIGMS MARC and MBRS Grants)

6.11

SELECTIVE LABELING OF HYPERREACTIVE SULFHYDRYL RESIDUES ON THE SR Ca^{2+} RELEASE CHANNEL WHICH ARE CRITICAL TO CHANNEL GATING. Guohua Liu*, Ildiko Zimanyi*, Jonathan J. Abramson*, and Isaac N. Pessah* (SPON: A.C. Bonham). Vet. Pharm. & Tox., University of California, Davis, CA 95616

Nanomolar concentrations of the fluorogenic maleimide 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM) was used to selectively label a single class of hyperreactive sulfhydryl (-SH) residues on the high molecular weight junctional foot (JFP) protein of the SR release channel. The number of -SH on JFP labeled by CPM decreased with the addition of channel activators in a dose-dependent manner. Labeling of JFP with nM CPM resulted in marked inhibition of [^{3}H]ryanodine binding and of Ca^{2+}-induced Ca^{2+} release from actively loaded SR vesicles and dramatically decreased the open probability (P_o) of a single channel in a bilayer. Under nonreducing conditions, fluorescence in SDS PAGE (4-20% gradient) gels loaded with CPM-labeled SR was selectively localized with the JFP monomer (M_r 340kDa). Under reducing conditions, however, the CPM-labeled JFP separated into M_r of 240 kDa and 100 kDa. These results provide direct evidence of the existence of critical thiols on the SR channel complex essential for normal channel gating. Intramolecular redox reactions between M_r 240 and 100 kDa appear to play an important role in gating of the channel. Supported by NIH ES05002.

6.12

$[Mg^{2+}]_o$ REGULATES INTRACELLULAR Ca^{2+} ($[Ca^{2+}]_i$) AND ITS SUBCELLULAR DISTRIBUTION IN CEREBRAL VASCULAR SMOOTH MUSCLE CELLS (VSMCs). Aimin Zhang*, Toni P.-O. Cheng*, Bella T. Altura and Burton M. Altura. SUNY, Health Science Center, Brooklyn, New York 11203

Changes in $[Ca^{2+}]_i$ under different extracellular Mg^{2+} concentrations ($[Mg^{2+}]_o$) were examined using the Ca^{2+} fluorescence indicator, fura-2, and digital-imaging fluorescence microscopy in single cultured canine cerebral VSMCs. $[Mg^{2+}]_i$ and external Ca^{2+} concentrations ($[Ca^{2+}]_o$) were determined by ion selective electrodes. Sucessive stepwise lowering of $[Mg^{2+}]_o$, by superfusion, from 1.36 mM to 1.07, 0.88, 0.48 and 0.17 mM resulted in significant rapid elevations of $[Ca^{2+}]_i$ in a concentration-dependent manner: $[Ca^{2+}]_i$ was increased from a basal level of 89.6±15.2 nM to 213.6±42.8, 560.1±81.8, 636.4±81.7 and 837.5±193.9 nM, respectively. Restoration of the $[Mg^{2+}]_o$ to 1.36 mM caused a fall in $[Ca^{2+}]_i$ to 215.9±52.3 nM, but only complete removal of $[Ca^{2+}]_o$ returned $[Ca^{2+}]_i$ to the initial level. Elevations in $[Ca^{2+}]_i$ occurred within a few minutes following changes in $[Mg^{2+}]_o$, and these did not seem to remain in a steady-state. Ca^{2+} oscillations were induced by reduction in $[Mg^{2+}]_o$ to 0.48 mM in the presence of $[Ca^{2+}]_o$. However, irrespective of the $[Mg^{2+}]_o$, a heterogeneous distribution of $[Ca^{2+}]_i$ was still evident in all cells tested, and the concentration of $[Ca^{2+}]_i$ in subcellular compartments increased concomitant with $[Ca^{2+}]_i$ when $[Mg^{2+}]_o$ was lowered. Perinuclear $[Ca^{2+}]_i$ was higher than either peripheral or nuclear $[Ca^{2+}]_i$. At 0.48 mM $[Mg^{2+}]_o$, peripheral $[Ca^{2+}]_i$, perinuclear $[Ca^{2+}]_i$ and nuclear $[Ca^{2+}]_i$ in some cells were increased 11-, 5- and 33-fold, respectively, when compared to 1.36 mM $[Mg^{2+}]_o$. These data suggest that: 1) canine cerebral VSMCs are very sensitive to $[Mg^{2+}]_o$; 2) changes in $[Mg^{2+}]_o$ can rapidly regulate subcellular $[Ca^{2+}]_i$ compartmentation, probably by modulating Ca^{2+} entry across the plasma membrane and its release from intracellular stores; 3) subtle, submillimolar changes in $[Mg^{2+}]_o$ may serve to act as acute, physiological regulators of ion transport in VSMCs. (Supported in part by ADAMHA, AA-08674.)

6.13

EXTRACELLULAR Mg REGULATES INTRACELLULAR FREE Mg ($[Mg^{2+}]_i$) AND ITS SUBCELLULAR COMPARTMENTATION IN CULTURED VASCULAR SMOOTH MUSCLE CELLS (VSMCs). Burton M. Altura, Aimin Zhang*, Toni P.-O. Cheng*, and Bella T. Altura. SUNY, Health Science Center, Brooklyn, NY 11203

Mg^{2+} is known to play an important role in regulation of Ca^{2+} metabolism and contractility of VSMCs. However, little is known about the regulation of Mg^{2+} homeostasis in these cells. The present studies, using digital imaging microscopy and the Mg^{2+} fluorescent probe, mag-fura-2, determined $[Mg^{2+}]_i$ and its subcellular distribution in single vascular smooth muscle cells (VSMCs) from rat aorta. With 1.2 mM $[Mg^{2+}]_o$ (control), $[Mg^{2+}]_i$ in VSMCs was 0.42±0.07 mM. Elevation of $[Mg^{2+}]_o$ up to 4.8 mM induced consistent increments in $[Mg^{2+}]_i$ (to mean values of 1.09±0.07 mM) in 5 min. Irrespective of the $[Mg^{2+}]_o$, a heterogeneous distribution of $[Mg^{2+}]_i$ was still evident in all cells tested. At 1.2 mM $[Mg^{2+}]_o$, perinuclear $[Mg^{2+}]_i$ (0.63±0.06 mM) was higher than either nuclear (0.45 mM±0.05 mM), or peripheral $[Mg^{2+}]_i$ (0.19±0.03 mM). The concentration of Mg^{2+} in subcellular compartments increased concomitant with $[Mg^{2+}]_i$ when $[Mg^{2+}]_o$ was elevated. With $[Mg^{2+}]_o$ at 4.8 mM, $[Mg^{2+}]_i$ concentrations of 2.73±0.2, 1.93±0.1 and 0.98±0.06 mM were found in perinuclear, nuclear and peripheral regions, respectively. These data suggest that rat aortic VSMCs are quite sensitive to $[Mg^{2+}]_o$ and that changes in $[Mg^{2+}]_o$ can regulate $[Mg^{2+}]_i$ and its subcellular compartmentation, which may be related to its inhibitory effects on intracellular Ca^{2+} concentration, vasoconstrictor agents, and basal tone of VSMCs. Lastly, these data seem to suggest that $[Mg^{2+}]_o$ may exert an important regulatory influence on intracellular membrane Mg channels. (Supported in part by ADAMHA, AA-08674.)

6.14

EXTRACELLULAR ATP INCREASES CYTOSOLIC CALCIUM IN RENAL VASCULAR SMOOTH MUSCLE CELLS(VSMC). E.W. Inscho, T.P. Belott*, M.J. Mason*, and L.G. Navar. Tulane Univ., New Orleans, LA. 70112

These studies were conducted to determine the ability of extracellular ATP to alter cytosolic calcium concentration in cultured renal arterial smooth muscle cells and to determine the mechanism by which this alteration occurs. VSMC were obtained by explant culture of minced rat renal arteries incubated in Medium-199 containing 10% fetal calf serum. Cells prepared for study were passaged and maintained on 12 x 18 mm glass coverslips. Changes in cytosolic calcium were determined by changes in the ratio of fura-2 fluorescence intensity at 340/380 nm (excitation) and 510 nm (emission). Cells were incubated in physiological salt solution (PSS) with 1.0 mM calcium or PSS containing no added calcium and 1.0 mM EGTA. 100 μM ATP caused the fluorescence ratio to increase from a control of 1.06 ± 0.05 to 2.06 ± 0.13 ($P < 0.01$) before stabilizing at 1.35 ± 0.04 (n=8; $P < 0.05$), indicating a sustained ATP-mediated elevation of cytosolic calcium. When extracellular calcium is reduced to near 0, the initial response to 100 μM ATP is attenuated with cell fluorescence increasing only from 1.16 ± 0.18 to 1.44 ± 0.18 (n=5) before rapidly declining to 1.02 ± 0.06. This would suggest that calcium release from an intracellular pool is involved in the initial rise in cytosolic calcium while extracellular calcium is required to sustain the increase. Thapsigargin was given to inhibit intracellular calcium ATPases and thereby deplete the intracellular calcium pool. Fluorescence increased from 1.34 ± 0.09 to 1.80 ± 0.13 (n=7; $P < 0.01$) after thapsigargin administration, suggesting inhibition of intracellular calcium uptake processes. Subsequent treatment with 100 μM ATP elicited no further change in fluorescence. Similar results were obtained with thapsigargin in nominally calcium free solutions. These data suggest that ATP-mediated increases in cytosolic calcium involve calcium release from the thapsigargin-sensitive, intracellular pool in conjunction with calcium influx from the extracellular medium.

6.15

INHIBITION OF Ca^{2+} TRANSPORT PATHWAYS BY ECONAZOLE, MICONAZOLE AND SKF 96365 IN RAT THYMIC LYMPHOCYTES.

M.J. Mason*, B. Mayer* and L.J. Hymel. Tulane Univ., New Orleans, LA 70112

Cytochrome P450 has been proposed to underlie the mechanism of regulation of the plasma membrane Ca^{2+} permeability by the Ca^{2+} content of the inositol (1,4,5) triphosphate-sensitive Ca^{2+}-pool. We have investigated the effects on divalent cation uptake in rat thymic lymphocytes of three structurally related imidazole reagents reported to inhibit redox mechanisms. Changes in $[Ca^{2+}]_i$ and $[Mn^{2+}]_i$ were measured fluorimetrically with indo-1 and/or quin2. Econazole, miconazole and 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SKF 96365) were found to be potent blockers of Ca^{2+} and Mn^{2+} uptake activated by release of Ca^{2+} from intracellular stores induced by the addition of thapsigargin. Additionally, we have found that concentrations of these agents required to abolish divalent cation uptake also induced a marked release of Ca^{2+} from the thapsigargin-sensitive intracellular stores, a result consistent with inhibition of the endosomal Ca^{2+}-ATPase. In agreement with this suggestion we have found that all three of these agents are potent inhibitors of isolated sarcoplasmic reticulum Ca^{2+}-ATPase. On the basis of these data we caution against the use of these compounds as selective inhibitors of cytochrome P450.

6.16

DEPLETION OF INTRACELLULAR Ca^{2+} STORES ACTIVATES BIPHASIC CHANGES IN MEMBRANE POTENTIAL IN RAT THYMIC LYMPHOCYTES.

O.I. Wilson , L.J. Hymel, M.P. Mahaut-Smith* and M.J. Mason*. Tulane Univ., New Orleans, LA 70112 and UCSD, La Jolla, CA 92093-0647

We have monitored changes in membrane potential (E_m) associated with Ca^{2+} influx stimulated by release of Ca^{2+} from intracellular stores in rat thymic lymphocytes. E_m was monitored using *bis*(1,3-diethylthiobarbituric acid)trimethine oxonol (*bis*-oxonol) in thymocytes loaded with 1,2-*bis*-(2-amino-phenoxy)ethane-N,N,N'N'tetraacetic acid (Bapta). Depletion of Ca^{2+}_i stores by the application of thapsigargin (33 nM), cyclopiazonic acid (5 μM) or ionomycin (20 nM) induced a depolarization which was dependent upon extracellular Ca^{2+}, abolished by 5 mM Ni^{2+} and independent of extracellular Na^+. This depolarization was found to temporally correlate with Ca^{2+} influx from the extracellular medium as measured fluorimetrically with indo-1. This depolarization was followed by a slow repolarization and subsequent charybdotoxin-insensitive hyperpolarization towards values approximating the K^+ equilibrium potential in these cells. In the absence of intracellular Bapta no significant depolarization was detected following the addition of thapsigargin, cyclopiazonic acid or ionomycin. These results demonstrate that depletion of Ca^{2+}_i stores induces biphasic alterations in E_m consistent with an increase in both the Ca^{2+} and K^+ conductance of the plasma membrane of Bapta loaded rat thymic lymphocytes.

6.17

DEVELOPMENTAL CHANGES IN CALCIUM ION TRANSPORT ACROSS THE CHICKEN ALLANTOIC MEMBRANE. **J. Blackburn, G. Grabowski*, Medical University of South Carolina, Charleston, SC 29425 and D. Silverthorn, University of Texas at Austin, Austin, TX 78712.**

The chicken allantoic membrane is an excellent model for studying developmental changes in sodium ion transport. Studies utilizing the Ussing chamber-voltage clamp technique have revealed that during the later stages of incubation (14 to 19 days), the short circuit current (SCC) is generated totally by the movement of sodium ions. However, J. Graves et al. (Am. J. Physiol. 251:C787-C794, 1986) observed that during the early stages of incubation (through 13 days) another ion is responsible for some of the SCC (~15-25%). In the present study, it was observed that amiloride (a drug which inhibits the apical movement of sodium ions) at a concentration of 10^{-4} M reduced the SCC to 19% of control values. The further addition of lanthanum chloride (which blocks the apical influx of calcium ions) at a concentration of 10^{-3} M resulted in a complete loss (and in some cases a change in polarity) of the positive SCC. Superfusion of the epithelium with bumetanide and 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) did not reveal any significant participation of anions in the generation of the SCC. All experiments were performed using Krebs--Henseleit solution (temp. 37 deg., pH 7.4) and a commercial Ussing chamber-voltage clamp system. The results of the present study indicate that calcium ions contribute to the SCC observed in the allantoic membrane of the embryonic chick during the early stages of development.

6.18

DITHIOTHREITOL INHIBITS CALCIUM TRANSIENTS BUT INDUCES MATURATION IN STARFISH OOCYTES. Harry J. Witchel, Dept. Physiology, Medical School, Bristol BS8 1TD, United Kingdom

Dithiothreitol (DTT), a nonspecific reducing agent, has been shown previously to be an agonist of starfish oocyte maturation.(1) DTT can both lower the threshhold dose for the membrane-acting matuaration hormone 1-methyladenine and can induce maturation by itself at a dose of 10 mM.(2) Recently it was shown that Thimerosal, a sulfhydryl reagent, can induce calcium transients in mouse oocytes.(3) In this communication we show that in oocytes of the starfish *Patiria miniata*, DTT inhibits a calcium transient that is normally induced by 1-methyladenine, that DTT does aid in hormonal induction of oocyte maturation, and therefore that the calcium transient seen after hormone stimulation (4,5) is not related to hormone transduction.

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6.19

INTRACELLULAR CALCIUM INCREASES AND OSCILLATES WITH THE FLAGELLAR BEAT CYCLE IN HYPERACTIVATED HAMSTER SPERM. S.S. Suarez, S.M. Varosi*, and X-B. Dai* (SPON: D.D. Buss). University of Florida College of Veterinary Medicine, Gainesville FL 32610.

Extracellular calcium is required for mammalian sperm to become hyperactivated, a movement pattern associated with attaining the capacity to fertilize. We used the fluorescent calcium indicator indo-1 to follow the relationship between intracellular calcium ([Ca]i) and the increased flagellar curvature characteristic of hyperactivation. Sperm (5 x 10^6/ml) were loaded with 10 μM indo-1AM for 40 min. Mn^{2+} quenched nearly all fluorescence in controls. The dye was excited at 340 nm by a xenon stroboscope producing 15 μsec flashes every 1/30 sec to provide crisp, sequential images from rapidly moving sperm. The emission was divided and delimited to bands surrounding peaks at 405 and 490 nm. Images obtained simultaneously at the 2 peaks were digitized and a mean ratio (R) was determined after subtracting background noise. A linear relationship was found between log[(R_{min})/(R_{max}-R)] and log[Ca] within the physiological range of [Ca]i. R was determined for 4 regions of each sperm: acrosomal, postacrosomal, proximal midpiece (half of the flagellar midpiece proximal to the head), and distal midpiece. R was higher in all regions of hyperactivated sperm than activated (fresh) sperm, indicating a higher [Ca]i (5 exp., 255 sperm, $p<0.001$)). [Ca]i correlated with flagellar curvature for all regions measured ($p<0.001$). In addition to this general increase, oscillations in [Ca]i were detected in all regions of individual sperm followed through several beat cycles. Fourier transformation of the data indicated that, for 11/16 hyperactivated sperm and 5/11 activated sperm, the frequencies for flagellar beat cycle and peak [Ca]i in the proximal midpiece were identical. Thus, the indo-1 emission patterns indicate that [Ca]i generally increases with increasing flagellar curvature and oscillates with the beat cycle. (NIH grant HD19854)

6.20

Na^+-Ca^{2+} EXCHANGE IN THE BASAL PLASMA MEMBRANE OF THE HUMAN PLACENTAL SYNCYTIOTROPHOBLAST. S. G. Kamath* and C. H. Smith. Depts. Pediatrics & Pathology, Washington Univ. Sch. Med. and Children's Hospital, St. Louis, MO 63110.

Mineralization of the fetal skeleton requires the placental transport of large quantities of Ca^{2+}. The need to maintain nanomolar Ca^{2+} concentrations within the syncytiotrophoblast and fetal Ca^{2+} concentrations greater than maternal suggests the presence of Ca^{2+} pumping mechanisms in the fetal facing basal plasma membrane. We have previously characterized an ATP-dependent Ca^{2+} pump at that location and now report the presence of a Na^+-Ca^{2+} exchanger. Isolated basal membranes were loaded with either K^+ or Na^+ and incubated with K^+ and $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ uptake was enhanced by an outwardly directed sodium gradient and the relative enhancement was maximal at approximately 10 min. The Na^+ ionophore monensin (5 μM) substantially inhibited Na^+-gradient stimulated ^{45}Ca uptake. Exit of $^{45}Ca^{2+}$ from vesicles preloaded for 6 sec was accelerated by extravesicular Na^+, providing further evidence for the presence of the exchanger. The presence of a Na^+-Ca^{2+} exchanger in addition to an ATP-dependent Ca^{2+} pump in the basal membrane suggests an increasing importance of cellular extrusion mechanisms in transporting Ca^{2+} into the fetal circulation.

6.21

NICOTINE STIMULATES Ca^{2+} INFLUX MEDIATED BY AN ACETYLCHOLINE RECEPTOR-OPERATED Ca^{2+} ENTRY PATHWAY IN RAT SUBLINGUAL MUCOUS CELLS. Guo H. Zhang* and James E. Melvin* (SPON: J. Arreola). Dept. of Dental Research, Univ. of Rochester, Rochester, NY 14642

The mechanism for Ca^{2+} influx in salivary acinar cells is still unclear. Recent studies have demonstrated a capacitative Ca^{2+} entry pathway activated by muscarinic receptor, distinct from L-type Ca^{2+} channels. In the present study, the effects of nicotine on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined using the Ca^{2+} sensitive fluorescent indicator, fura-2. Nicotine induced a dose-dependent increase in $[Ca^{2+}]_i$(3-fold maximal), which was completely prevented in a Ca^{2+}-free medium, indicating that the increase results from Ca^{2+} influx and not Ca^{2+} release from intracellular store. Inhibitors of capacitative Ca^{2+} entry (La^{3+} and Ni^{2+}) did not affect the nicotine-induced Ca^{2+} influx; whereas, the L-type Ca^{2+} channel blockers, diltiazem (10 μM) and D888 (10 μM) inhibited 66% and 96% of Ca^{2+} influx, respectively. Furthermore, inhibitors of the nicotine acetylcholine receptor, decamethonium (50 μM), hexamethonium (10 μM), and tubocurarine (10 μM), blocked 77%, 90%, and 73% of Ca^{2+} influx, respectively, without affecting the muscarinic-stimulated $[Ca^{2+}]_i$ increase. Taken together, these results strongly suggest that in sublingual acinar cells there is an acetylcholine receptor-operated Ca^{2+} entry mechanism. (Supported by Smokeless Tobacco Research Council project #381-01 and NIH/NIDR R01 DE08921.)

6.22

DOSE DEPENDENT EFFECTS OF RYANODINE ON THE MEMBRANE POTENTIAL OF CARDIAC PURKINJE TISSUE. Kiernen Seth, Shukla Sarker, and M.L.Bhattacharyya. Meharry Medical college, Nashville,TN.37208

We tested the effects of Ryanodine on the membrane potential and action potential (AP) of a driven (1Hz) Purkinje tissue or an overdriven (3Hz) normally spontaneously beating Purkinje tissue. In overdrive (OD) experiments the driving rates were increased to 2-3Hz for 1-2min, in various test solutions to see the post drive (PD) activity (suppression or excitation). Ryanodine prolonged the action potential duration at all levels in normally driven (1Hz) preparations(10^{-9} - 10^{-6}M), flattened the diastolic depolarization (DD) region of the AP in a dose dependent manner (10^{-7} - 10^{-6}M). At 10^{-7}M, the maximum diastolic potential (MDP) was slightly more negative than that at the control (no Ryanodine). Ryanodine at higher doses (10^{-6}M), increased the slope of the DD, depolarized membrane potential (10-15 mV), and in time (20-25 min) extrasystoles were seen. Overdrive experiments constantly show a suppressed PD activity (10^{-9} - 10^{-7}M) and a single large post drive transient oscillatory potential (the amplitude of which diminished in subsquent later drives) at higher concentration (10^{-6}M). These effects on AP parameters and post drive membrane potential suggest that Ryanodine may have effects on plasma membrane channels besides its effects on calcium release channels of the sarcoplasmic reticulum, and that Ryanodine at higher concentration does not prevent calcium overloading of the cytoplasm.

6.23

THE MECHANISM OF EARLY GLUCOCORTICOID FEEDBACK DIFFERS IN MOUSE PITUITARY TUMOR CELLS AND SHEEP ANTERIOR PITUITARY CELLS. Terrence P. Clark, Marlon B. Slater*, James L. Sartin, Robert J. Kemppainen. Department of Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Alabama 36849.

Glucocorticoid negative feedback on stimulated adrenocorticotropin (ACTH) secretion at the anterior pituitary can be divided into fast, intermediate, and slow time domains. Fast and intermediate time domains are often referred to as early feedback when studied *in vitro*. It has recently been shown that glucocorticoids modulate the influx of extracellular calcium in AtT-20 cells suggesting that steroids may block a voltage-dependent calcium channel. In order to determine if mouse pituitary tumor cells are a representative model for early glucocorticoid inhibition in normal corticotrophs, we contrasted the effects of glucocorticoids on AtT-20 cells with enzymatically dispersed sheep anterior pituitary (AP) cells *in vitro*. Sheep AP cells were 100-fold more sensitive to dexamethasone (DEX) inhibition of corticotropin-releasing hormone (CRH)-stimulated ACTH release compared with AtT-20 cells. Furthermore, dexamethasone inhibited CRH-stimulated ACTH secretion from sheep AP cells by 51% while the inhibition was only 32% in AtT-20 cells. Dexamethasone (10^{-7} M for 2 hours) significantly inhibited KCl-stimulated ACTH release from sheep AP cells, but not in AtT-20 cells. Similarly, inhibition of maitotoxin (MTX)-stimulated ACTH release was present in sheep AP cells but not in AtT-20 cells. However, in both cell types CRH, KCl, and MTX-stimulated ACTH release was inhibited in a dose dependant manner by the dihydropyridine calcium channel blocker nifedipine. These data suggest that glucocorticoids may inhibit stimulated ACTH release through a calcium sensitive mechanism in sheep AP cells but not in AtT-20 cells. Therefore, AtT-20 cells may not be representative of normal corticotroph function with regard to early glucocorticoid negative feedback.

9.1

ESTRADIOL INCREASES EXTRACTION OF ^{14}C-2-DEOXYGLUCOSE ACROSS THE BLOOD-BRAIN BARRIER. J. Bishop* and J.W. Simpkins. Center for the Neurobiology of Aging, University of Florida, Gainesville, FL 32611.

We have previously shown that physiological doses of estradiol benzoate (E_2B) increased cerebral glucose utilization by 20 to 120% when compared to oil-treated ovariectomized rats. These data are confirmed with autoradiographic studies which revealed estradiol increases glucose utilization in the CNS in a manner that was not confined to brain regions which contained estradiol receptors. We pursued these observations using the Oldendorf method to measure the effect of E_2B on brain uptake of ^{14}C-2-deoxyglucose (^{14}C-2-DG) across the blood-brain barrier (BBB). This method compared the extraction of a tracer sugar, ^{14}C-2-DG, with an internal standard of tritiated water, (^3H_2O). Our results showed that E_2B (10μg s.c. in oil, 4 hours exposure) increased extraction of the labeled sugar across the BBB by 45% when compared to oil-treated controls. The efflux rate constants for ^{14}C-2-DG, ^3H_2O, or the estimate of cerebral blood flow did not change. The values agreed with prior literature. These data demonstrate that a single dose of estradiol significantly increased the extraction of ^{14}C-2-DG across the BBB. The data also raises the possibility that estrogens may play an important and physiological role in brain glucose homeostasis. (Supported by AG 10485 and NS 07333)

9.2

INOSITOL TRANSPORT IN CARDIAC SARCOLEMMAL VESICLES. Leona J. Rubin and Calvin C. Hale. Univ. of Missouri, Columbia, MO 65211.

Myo-Inositol is the precursor for synthesis of inositol-containing phospholipids involved in the inositol phosphate signal transduction pathway. Within the myocardium, basal levels of either or both inositol phospholipids or inositol phosphates may be altered during chronic pathophysiological conditions such as diabetes or endotoxemic shock. However, little is known about the inositol transporter within the myocardium, the principal supplier of cytoplasmic *myo*-inositol. Utilizing bovine sarcolemmal (SL) vesicles, we have examined the process of inositol transport within the myocardium. Cardiac SL vesicles were prepared from left ventricular tissue and equilibrated overnight at 4°C in an isosmotic KCl buffer. ^{3}H-*myo*-inositol incorporation in SL vesicles was both a time- and Na^+-dependent process. Transport was stimulated 3.5-fold by addition of 1μM valinomycin suggesting the process is also electrogenic. In addition, *myo*-inositol transport was sensitive to Mn^{2+} and Mg^{2+} but not Ca^{2+}. Peak transport of *myo*-inositol occurred at 0.5mM Mn^{2+} with decreased activity at higher concentrations (1-10mM). Maximal stimulation by Mg^{2+} was at 1 mM and was unchanged at higher (up to 10 mM) concentrations. Kinetic analysis revealed an apparent high and low affinity transport process. High affinity *myo*-inositol transport was saturable demonstrating a Vmax of 0.7 ± 0.07nmoles inositol/mg protein/min and a Km of 39 ± 2μM. Low affinity inositol transport was unsaturable up to 1.0 mM substrate. *Myo*-inositol transport was not inhibited by 1mM of either L-*chiro*-inositol, D-*chiro*-inositol, L-glucose, or D-glucose. *Scyllo*-inositol at 1mM, however, inhibited transport nearly 100%. Kinetic analysis established *scyllo*-inositol as a competitive inhibitor of cardiac *myo*-inositol transport, 50μM *scyllo*-inositol increased the Km to 106 ± 21μM. These data indicate that myocardial inositol transport is a Na^+ co-transport process that is electrogenic, cation stimulated, and stereospecific. Supported by AHA (LJR) and AHA-Missouri Affiliate (CCH).

9.3

BASOLATERAL, SODIUM DEPENDENT INOSITOL TRANSPORT ON EPITHELIAL THYROID CELLS. Mauchamp Jean, Verrier Bernard and Coulomb Cyril, *INSERM U270* Faculté de Médecine Nord, Bld P. Dramard F-13326 Marseille Cedex 15 (France).

The free inositol content of the thyroid gland is one of the highest among all organs. We have studied the properties and localization of inositol uptake by cultured porcine thyroid cells. When cultured cells were organised into follicular structures, the basolateral surface oriented towards the medium, they actively concentrate inositol present in the culture medium. An almost complete depletion of medium inositol was obtained within 12 hours. More than 90 % of cellular inositol was free. Inositol uptake was sodium dependent with a Km for inositol of 0.13 mM. It was inhibited by inosose -2.

When cells are organized into monolayer on a plastic substrat, apical pole towards the culture medium, inositol uptake was not observed.

Finally when cells were cultured on the porous bottom of bicameral culture chambers (Costar 0,3μm) they formed a tight electrically active polarized monolayer. The sodium dependent inositol concentration mechanism was exclusively (92 %) present on the basolateral surface of the monolayer.

The sodium dependent inositol transport mechanism present on the apical surface of kidney and intestinal epithelium is therefore located at the basolateral surface on the thyroid epithelium.

9.4

TOPOGRAPHY OF THE RABBIT RENAL SODIUM-D-GLUCOSE COTRANSPORTER EXPRESSED IN XENOPUS OOCYTES. A.I.Morrison-Shetlar*, R.Shetlar*, M.Panayotova-Heiermann*, F.Pietruschka*, R.K.H. Kinne. Max-Planck Institut für Systemphysiologie, Dortmund 1, FRG.

To test a recently proposed model for the renal sodium-D-glucose cotransporter four monoclonal antibodies were raised against specific peptide sequences (P4- amino acids 624-634, P3- 236-249, P2- 97-111, P1- 57-73). Xenopus oocytes injected each with 50ng cRNA coding for the cotransporter or an equivalent volume of water were incubated for 5-6 days at 18oC before being used for ^{14}C-alpha methyl glucose (^{14}C-AMG) uptake studies or for histological studies employing fluorescein labelled secondary anti-mouse antibodies and confocal laser scanning microscopy. Transport studies indicated that the injected cRNA stimulated ^{14}C-AMG uptake by about 300 fold (H_2O injected: 0.17 ± 0.05 pmol/oocyte/hr; cRNA injected: 52.0 ± 12.7), >90% of the uptake was inhibited by 0.1mM phlorizin. Histological studies showed strong binding of P1, P2 and P4 to the oocyte and 350um optical sections indicated only membrane bound fluorescence in both non-permeabilized and permeabilized cells. P3, but not P1, both postulated to be localized on the cytoplasmic side of the membrane, was negative in non-permeabilized cells but strongly positive in permeabilized cells. The rabbit renal Na-D-glucose cotransporter has the following topography: P4 (carboxyl end of the protein) is, as in the Na-proline cotransporter, located extracellularly as are P3 and P1 while P2 lies cytoplasmically.

9.5

THE ROLE OF GLUT2 IN THE TRANSPORT OF FRUCTOSE ACROSS THE INTESTINAL BASOLATERAL MEMBRANE. Chris Cheeseman, Dept. of Physiology, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

Fructose is taken up into intestinal epithelial cells by a sodium independent mechanism, but its route of exit into the bloodstream has not been fully characterized. GLUT2 is a facilitated glucose transporter isoform found in the intestinal basolateral membrane (BLM) and functional expression in oocytes indicates that it can carry both glucose and fructose. Rat jejunal isolated basolateral membrane vesicles were employed in this study to characterize fructose transport. Uptake was found to be sodium-independent with a km of 13.4 mM and a Vmax of 787 pmoles/ mg prot./ sec. Saturable uptake was abolished by 100μM cytochalasin B and could be inhibited by D-glucose, 3-O-methyl-D-glucose, 6-Deoxy-D-glucose, and D-mannose. α-methyl-D-glucopyranoside, L-glucose and sucrose had no effect on fructose uptake. Cross inhibition experiments showed that fructose and glucose could mutually inhibit each other's uptake 100%. Conditions known to increase glucose transport across the BLM; luminal perfusion *in vivo* for 4 hours with 100mM D-fructose or feeding a high carbohydrate diet for one week, increased fructose transport in parallel with that of glucose. It is concluded, therefore, that GLUT2 alone is responsible for moving both glucose and fructose out of the enterocyte across the BLM. The potentiating effect of luminal glucose on fructose absorption seen *in vivo* can now be explained by the modulation of GLUT2 activity in the BLM and does not require an unexplained mechanism at the apical pole of the epithelium.

Supported by the CDA and MRC Canada

9.6

MOLECULAR EVIDENCE FOR TWO DIFFERENT NA-D-GLUCOSE COTRANSPORTERS (NA-D-GLC) IN DOG KIDNEY CORTEX. M. Silverman, P. Speight and L. Ho. Univ. of Toronto, Toronto, Ont.

In brush border membrane vesicles (BBMV) from outer cortex (OC) of rabbit kidney there is a low affinity Na-D-glC with 1:1 Na:D-gl stoichiometry and in outer medulla (OM) a high affinity system with stoichiometry of 2:1 (AJP 242:F406, 1982). A >99.9% homologue of SGLT1 (Nature 330:379,1987) has been cloned from rabbit renal cortex (AJP 259:C605,1990). Recently a new low affinity (SGLT2) Na-D-glC has been cloned and expressed in xenopus oocytes (Hediger, personal communication). We prepared BBMV from OC and OM of dog kidney and studied the kinetics, Na:D-gl stoichiometry and probed both preps with two polyclonal antibodies (Ab) raised against peptides from specific a.a. sequences of SGLT1, 402-420 (AbE generous gift of E. Wright) and 565-574 (AbP) prepared in our lab. OC BBMV show a low affinity Na-D-glC Km of 5.98±1.0mM (40mM Na gradient, $\Delta\psi$ = 0) Na:gl 1:1. In OM the Km is 0.27± 0.03mM and Na:D-gl 2:1. On Western blots both AbE and AbP bind specifically to two polypeptides of 72.5 and 75.5 KDa. The 72.5 KDa band is enriched in OM (4:1) and conversely the 75.5 KDa band is enriched in OC (13:1). This result cannot be explained by proteolysis. We conclude that (1) the 72.5 KDa protein enriched in OM is SGLT1 and the 75.5 KDa protein in OC is SGLT2, and (2) SGLT1 and SGLT2 are homologous for AbE and AbP. This is the first evidence to localize SGLT1 and SGLT2 in situ to distinct regions of the renal cortex.

9.7

EFFECTS OF DIETARY Na INTAKE ON SMALL INTESTINE AND KIDNEY OF DIABETIC RATS. M. Qulali*, H. Feister*, K. Camp*, L. Maianu*, and J.H. Dominguez* (SPON: T.D. McKinney). VAMC & IUMC, Indianapolis, IN, 46202.

Diabetes induces overexpression of GLUT 2 glucose transporter and also enlargement in renal proximal tubule (PT). These changes may compensate for the collapsed glucose gradient observed in hyperglycemia, and increase glucose flux (ASN91). Small intestine (SI) which also transports glucose vectorially, may undergo changes in streptozotocin-induced diabetic rats (D). Therefore, the hypothesis that diabetes enhanced SI size and GLUT 2 gene expression was tested. Five weeks post-induction of diabetes, D rats were hyperglycemic (478 ± 8 mg/dl, n = 10, $p<0.05$), while glucose in control (C) rats = 96 ± 3, and in insulin-treated diabetic (D&I) rats = 68 ± 9. SI was enlarged in D (48 ± 3 inches, n = 11, $p<0.05$) over C (40 ± 2) or D&I (37 ± 2). GLUT 2 mRNA and protein levels were increased in duodenum and jejunum of D rats. We then tested if increase in solute (glucose/sodium) flux participated in gene expression changes in C and D rats 3 weeks post-induction of diabetes. Rats were pair-fed (loNa PF) or fed ad lib (loNa adlib) a low Na diet. We found that SI and kidney sizes increased in D loNa adlib, similar to D rats fed normal Na diet, but in marked contrast, kidney and SI sizes did not increase in D rats fed loNaPF. In conclusion: (i) like PT, GLUT 2 in SI is also overexpressed in D rats, which may increase glucose flux in hyperglycemia. (ii) In D rats organomegaly may be regulated by solute ingestion, and may be prevented by reducing solute load.

9.8

GLUCOSE-DEPENDENT EXPRESSION OF GLUT 1 IN 3T3-L1 ADIPOCYTES. H. Kitzman Jr.*, R. McMahon*, M. Williams*, and S. Frost* (SPON: M.S. Kilberg). Univ. of Florida, Gainesville, FL 32610

Elevated glucose transport rates during glucose deprivation is a phenomenon that has been observed in several different types of cells in culture. We show here that glucose transport rates in 3T3-L1 adipocytes increased by 10-fold within 18h in response to glucose deprivation. Mannose and 3-O-methylglucose (a non-metabolizable glucose analog), but not fructose or galactose, blocked the increase in transport activity. Although the increase in transport was dependent on new protein synthesis, only a small and transient increase in GLUT 1 mRNA (less than 2-fold) was observed. In addition, the level of the normal isoform of GLUT 1 (46kDa) did not increase. A lower molecular weight isoform (37kDa) was observed but not until 15h after glucose removal, the appearance of which was clearly not correlated with the increase in activity. This form (p37) was slightly larger than N-glycosidase F-treated GLUT 1 (36kDa), implying that the p37 form is still glycosylated, albeit incompletely. Like transport activity, the appearance of p37 was dependent on new protein synthesis. Interestingly, p37 was seen in the presence of galactose, but not fructose, despite elevated transport activity with either sugar. Thus the time- and sugar-dependent appearance of the lower isoform suggests that p37 is not responsible for starvation-induced transport but potentially represents an underglycosylated precursor of the normal, 46kDa isoform of GLUT 1. (NIH:DK45035)

9.9

INSULIN STIMULATION OF GLUCOSE TRANSPORT IN VASCULAR SMOOTH MUSCLE CELLS: POSSIBLE IMPLICATION OF GLUT-4 TRANSPORTER IN THE VASCULATURE. **Paul R. Standley*, Kelly Rose*, Prakash Turiputur*, and James R. Sowers * (SPON: Jeffrey L. Ram). VA Medical Center and Wayne State University, Detroit, MI 48201**

We have previously reported that vascular smooth muscle cells are insulin sensitive. Insulin 1) attenuates Ca^{2+} responses to voltage and vasoactive stimuli in VSMC, 2) decreases contractility of blood vessel segments, and 3) increases Na^+K^+ATPase activity and transcription of the alpha-2 subunit of this pump in VSMC. Thus, insulin may serve to modulate VSMC tone, and thus blood pressure. In order to test whether VSMC are insulin (INS) sensitive in the "classical" or metabolic sense, we investigated the effects of insulin on ^{3}H-2-dexyglucose (DOG) transport into cultured VSMC. Three rat cell lines were used: a7r5 VSMC (aortic), tail artery VSMC (Sprague Dawley), and aortic VSMC derived from the Zucker lean rat. Cells were cultured to confluence, acclimated with PBS, pH 7.4 at 37°C for 60 min and then pretreated with or without INS for 20 min. Timed studies revealed significant increase in DOG uptake after 5 min with 700 nM INS with all cell lines ($p<0.05$, n+3-8). Dose response studies (30 min DOG uptake) in both aortic cell lines revealed that the lowest [INS] needed to elicit a significant increase in DOG uptake was approximately 2.1 nM, while maximum stimulation occurred at 700 nM ($p<0.01$ MANOVA, n=2). The estimated $ED_{\frac{1}{2}}$ for insulin's effect was approximately 40 nM. Incubation with 2.5 pg/ml of a polyclonal INS receptor Ab to the alpha subunit significantly increased DOG uptake by 20% ($p<0.05$), suggesting that INS effects are mediated, at least in part, by the INS receptor specifically. In addition, preliminary data using primers for exon 9 of glucose transporter 4 (GLUT4) and polymerase chain amplification of reverse transcribed mRNA extracted from a7r5 VSMC revealed that GLUT4 is transcribed in these cells. In summary, this is the first evidence that glucose uptake in VSMC is stimulated by INS. This effect may be mediated by the GLUT4 system. These data lend increasing evidence that VSMC are an insulin sensitive tissue, a property which has been shown to be implicated in blood pressure control.

9.10

mRNA QUANTIFICATION FOR RAT BRAIN GLUCOSE TRANSPORTER (GLUT1), USING MODIFIED "PCR AIDED TRANSCRIPT TITRATION ASSAY (PATTY)" METHOD.. K.C. Wadhwani, R. Fukuyama*, K. Chandrasekaran*, Q.R. Smith and S.I. Rapoport. Lab. Neurosciences, NIA, NIH, Bethesda, MD, 20892.

Changes in levels of mRNA of glucose transporters have been observed under various physiological and pathological conditions. In order to quantitate such changes, we tested whether the PATTY method (Nucl. Acid. Res., 17:9437, 1989) of mRNA determination would be useful. A 405bp [^{3}H]-labelled riboprobe containing the coding and 3'- noncoding sequences of the glucose transporter GLUT1 was prepared from rat cDNA plasmid (prGT3). A known amount (400 pg) of this synthetic [^{3}H]-RNA was reverse transcribed using random primers. The cDNA was then amplified using 2 primers corresponding to the noncoding sequences of GLUT1. This resulted in the amplification of a 292 bp fragment specific to the rat GLUT1 gene (target DNA). An oligo nucleotide of 100 bp containing the same sequences as the first and last 50 bases of the 292 bp fragment was also prepared (competitive DNA). Identical amounts of 'target DNA' were then coamplified with different amounts of the 'competitive DNA' (ranging from 1 pg to 24 ng) using the same primers. The 5'- primer was end labelled with ^{32}P. The ratio of radioactivity between the 'target DNA (292 bp fragment)' and 'competitive DNA (100 bp fragment)' was determined quantitatively after separation by gel electrophoresis and radioactivity counting. The method gave an accurate determination of the amount of synthetic [^{3}H]-RNA of GLUT1 transporter and allowed detection of a 2-fold changes in the amounts of synthetic RNA. Using this method, we estimate that the level of the rat GLUT1 mRNA in the isolated cerebral micro-blood vessels of the rat brain is 10 pg/μg total RNA , and is a 20-fold higher than that in PC12 cell line.

9.11

CYSTINE TRANSPORT IN RAT ALVEOLAR TYPE II CELLS. S.M. Deneke*, D.M. Bukowski*, R.A. Lawrence* and S.G. Jenkinson* (SPON: R.J. King). Univ TX Hlth Sci Ctr-SA & Audie L. Murphy VA Hosp, San Antonio, TX 78284.

Cystine transport in type II alveolar epithelial cells from adult rats grown for 24-48 hr on plastic was not induced by treatment with various agents, including arsenite and diethylmaleate (DEM), which induce cystine transport in pulmonary artery endothelial cells, mouse peritoneal macrophages or human diploid fibroblasts. We have characterized cystine transport in type II cells and found that the transport system is quite distinct from the x^-_c system first described by Bannai and Kitamura (J Biol Chem 255:2372, 1980) in fibroblasts. Unlike the x^-_c system, cystine transport in type II cells is primarily sodium dependent. Cystine uptake in type II cells is inhibited by the sodium-potassium ATPase inhibitor ouabain, but not affected by the sodium channel blocker amiloride. The inhibition of transport of cystine into type II cells by competitive amino acids also differs from properties of x^-_c-like systems reported in other cell types. In fibroblasts and type II cells glutamate is a strong competitive inhibitor and homocysteate is even more effective. Aspartate is not transported by the x^-_c system and does not significantly inhibit uptake of cystine by the x^-_c system. By contrast, in type II cells aspartate inhibited cystine uptake more effectively than either glutamate or homocysteate. Cysteine transport in type II cells was generally similar to other cysteine transport systems described.

9.12

EXPRESSION CLONING OF A HUMAN cDNA FROM KIDNEY CORTEX THAT INDUCES TRANSPORT OF L-CYSTINE IN *XENOPUS* OOCYTES. A. Werner"""*, J. Bertran"*, J. Chillarón"*, V. Nunes"""*, J. Biber"""*, X. Testar"*, A. Zorzano"*, X. Estivill"""*, H. Murer""" and M. Palacín"*.
"Dept. de Bioquímica i Fisiologia, Universitat de Barcelona, 08028; ""Dept. de Genètica Molecular, Institut de Recerca Oncològica, Barcelona, 08908, Spain."""Physiologisches Institut, Universität Zürich, 8057, Switzerland.

A recently isolated kidney cortex cDNA clone (rBAT) induces in *Xenopus* oocytes system $b^{o,+}$-like amino acid transport activity (J. Bertran et al., Proc. Natl. Acad. Sci. (USA) 89: 5601-5605, 1992). Expressed uptake relates to a single component of sodium-independent transport for dibasic, some neutral amino acids and L-cystine. The rBAT mRNA is found mainly in kidney and intestinal mucosa. rBAT might be involved in human cystinurias, genetic diseases that result in abnormalities of intestinal and renal absorption of cystine and dibasic amino acids. As a first step to study this hypothesis we have isolated a cDNA clone (about 2.4 kb) by screening a human kidney cortex cDNA library for expression of L-arginine transport in *Xenopus* oocytes. This cDNA induces in oocytes sodium-independent transport of L-arginine, L-leucine and L-cystine, via the system $b^{o,+}$ activity induced by rBAT. This cDNA codifies for a membrane protein since (^{35}S)-methionine labelling of oocytes shows a specific band of 90 kDa in crude oocyte membranes. Initial sequencing steps show a high level of amino acid sequence identity to the rabbit rBAT (65% and 70% identity for the 90 N-terminal and 10 C-terminal amino acid residues, respectively). We conclude that we have isolated a functional cDNA corresponding to the human rBAT.

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9.13

MULTIPLE COMPONENTS OF MEDIATED L-GLUTAMINE (L-GLN) TRANSPORT IN RAT CARDIAC MYOCYTES. L.J. Van Winkle, D.J. Paulson, G.T. Needham*, J.G. Liesen*, A.L. Campione* and D.F. DiDomenico*, Departments of Biochemistry and Physiology, Chicago College of Osteopathic Medicine, Downers Grove, IL 60515.

Net L-Gln flux into or out of skeletal muscle cells may help control the rate of net protein synthesis or degradation in this tissue. The importance of Gln transport to protein and amino acid homeostasis has not, however, been investigated thoroughly in cardiac muscle. Since heart appears not to be spared from the nitrogen loss associated with severe injury or infection, we have begun to characterize Gln transport in rat cardiac myocytes. Mediated Gln transport was inhibited more than 50% by 5-10 mM L-Asn, L-His, L-azaserine, L-glutamate γ-hydroxamate, and N^{π}-methyl-L-histidine. Transport was inhibited less than 50% by L-Leu, L-Ala, L-Ser, L-Tyr (2mM), L-DON, and L-DONV, and it was not inhibited in a statistically significant manner by MeAIB, BCH, BCO, L-Arg, L-Glu or N^{τ}-methyl-L-histidine. The component of transport that was inhibited by L-Leu also appeared to be inhibited by L-Ala, and L-Ala may have inhibited an additional component of Gln transport that was not inhibited strongly by L-Leu. Inhibition of transport by L-Ala plus N^{π}-methyl-L-histidine was greater than inhibition by either substance alone. These data are consistent with the possibility that system N and two other systems, but not systems L or A, transport Gln in rat cardiac myocytes.

9.14

CHARACTERIZATION OF A NOVEL SODIUM-INDEPENDENT PLASMA MEMBRANE GLUTAMINE (GLN) TRANSPORT SYSTEM IN RAT LIVER. Anthony J Pacitti* and Wiley W Souba, Dept of Surgery, University of Florida, Gainesville, FL 32610.

In hepatocyte plasma membrane vesicles (HPMVs) from rat liver, we observed that nearly half of Na^+-independent glutamine (GLN) uptake occurs by a saturable, carrier-mediated process with a Km = 1.15 mM and a Vmax = 240 pmol/mg protein/10 sec. This component of GLN uptake is mediated by a transport agency distinct from that of previously described Na^+-independent carriers. The model System L substrate BCH (2-amino[2,2,1]-heptanedicarboxylic acid) showed no appreciable inhibition of Na^+-independent GLN uptake by HPMVs, but effectively inhibited the uptake of leucine, a classic System L substrate. Further evidence against System L-mediated GLN transport was provided by the pH dependence of this carrier and the lack of trans-stimulation of saturable uptake. Competition experiments with selected amino acids revealed a pattern of inhibition of GLN transport which was inconsistent with assignment of glutamine entry to System asc, T, or systems for the Na^+-independent transport of the changed amino acids. This novel Na^+-independent transport system in HPMVs was highly selective for glutamine, histidine, and, to a lesser extent, asparagine. The slight inhibition of Na^+-independent GLN transport by leucine was non-competitive in nature. Based on the Na^+-independence, pH sensitivity, absence of trans-stimulation, and an amino acid selectivity similar to that of the previously described hepatic Na^+-dependent System N, we have provisionally designated this Na^+-independent GLN transporter in rat liver as System n.

9.15

EXPRESSION OF Na^+-DEPENDENT GLUTAMATE UPTAKE IN *Xenopus* OOCYTES USING mRNA FROM DEXAMETHASONE-TREATED RAT LIVER. P.M. Taylor*, B. Mackenzie*, E. Robertson* & M.J. Rennie. Department of Anatomy & Physiology, University of Dundee DD1 4HN, Scotland UK.

Treatment of rats with dexamethasone (0.44mg/kg/day over 8 days) induces Na^+-dependent uptake of the anionic amino acid glutamate (system X^-_{ag}) in sinusoidal membrane of liver (Low et al, *Biochem J* **284**: 333-340, 1992). *Injection* of oocytes with 50ng mRNA (in 50nl water) isolated from such livers resulted after 6 days in the expression of a Na^+-dependent component of glutamate uptake (0.149±0.051 pmol/oocyte.min) which was absent in oocytes injected with tRNA/water (n=15-17 oocytes: mean±sem). For each oocyte, uptake of 0.01mM radioactive glutamate was measured in 100mM NaCl (using [^{14}C]-tracer) and 100mM tetramethylammonium-Cl (using [^{3}H]-tracer) buffers consecutively (applying a correction for tracer efflux as appropriate); Na^+-dependent uptake was calculated as the difference between uptake in the two buffers. Injection of liver mRNA also stimulated Na^+-independent glutamate uptake in oocytes (from 0.097±0.021 to 0.367±0.071 pmol/oocyte.min) without a corresponding increase in Na^+-independent uptake of the neutral amino acid glutamine. These preliminary results indicate an opportunity to develop a strategy for expression-cloning of liver glutamate transporters.

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9.16

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF HUMAN BRAIN GABA TRANSPORTER. Hemanta K. Sarkar (SPON: D.L. Kunze). Baylor College of Medicine, Department of Molecular Physiology and Biophysics, Houston, Texas 77030.

The human brain GABA transporter cDNA clone (hGAT-1; Nelson, *et al.*, *FEBS Lett.* **269**:181-184, 1990) was subcloned into the multiple cloning site (MCS) of an expression vector containing T7 promoter/MCS/poly A tail/T7 terminator. *In vitro* transcribed RNA (cRNA) prepared from the resultant recombinant plasmid pVE.HGAT (containing the hGAT-1 insert in the right orientation under T7 promoter control) induced a Na^+ and Cl^- dependent [^{3}H]-GABA uptake activity in *Xenopus* oocytes which were microinjected with the cRNA. The uptake activity was significantly inhibited by nipecotic acid (~65% by 10 μM), moderately by β-ala (~25% by 100 μM) and in contrast to the rat brain GABA transporter (~50% inhibition by 100 μM L-DABA; Guastella, *et al.*, *Science* **249**:1303-1306, 1990), was least affected by L-DABA (no inhibition by 100 μM; ~20% inhibition by 500 μM). Further examination of GABA uptake inhibition using three glial GABA transporter specific inhibitors (*cis*-4-hydroxynipecotic acid, THPO and Guvacine; Schousboe, *et al.*, *Epilepsia* **24**:531-538, 1938) revealed that all three of them were able to inhibit the cRNA induced GABA uptake activity in microinjected oocytes with IC_{50} close to that of the glial type GABA transporter (Schousboe, *et al.*, 1983). These results suggests that the GABA uptake activity induced by hGAT-1 in *Xenopus* oocytes is of the glial type. [Supported by AHA, Texas Affiliate and Gordon and Mary Cain Pediatric Neurology Research Foundation].

9.17

ONTOGENY OF AMINO ACID TRANSPORT SYSTEM L IN BASOLATERAL AND CANALICULAR MEMBRANE VESICLES DERIVED FROM RAT LIVER. Donald A. Novak*, Mark Beveridge* and Michael S. Kilberg. Univ. of Florida, Gainesville, FL 32610-0296

Sodium-independent transport of branched chain amino acids into the hepatocyte occurs via at least two distinct transport processes, System L_1, characterized by low Km values, predominates in hepatoma and fetal cells, while System L_2, distinguished by Km values in the mM range, predominates in adult hepatocytes. System L activity in primary rat hepatocyte cultures is sensitive to inhibition by N-ethylmaleimide (NEM); that in hepatoma cell lines is not. In order to determine the membrane sub-domain distribution and ontogeny of System L in the hepatocyte we prepared highly enriched basolateral (BLMV) and canalicular (CMV) membrane vesicles from the livers of suckling (10 d/o) and adult rats. The initial rate uptake of [^{3}H]-leucine into BLMV and CMV derived from adult liver was significantly inhibited by the addition of 5mM NEM; transport into BLMV and CMV derived from 10 d/o liver was not affected. Michaelis-Menton kinetic parameters estimated in BLMV derived from adult liver were consistent with System L_2; Km = 5.84 ± 0.54 mM, Vmax = 1623 ± 56 pmol·mg^{-1} protein·5 sec^{-1}. We conclude that NEM inhibitible, sodium-independent leucine transport activity consistent with System L_2 is present in both basolateral and canalicular membrane vesicles derived from adult but not 10 d/o rat liver.

9.18

PHORBOL ESTER-INDUCED MEMBRANE PROTEINS IN CHRONIC LEUKEMIC B LYMPHOCYTES: CANDIDATE PROTEINS FOR THE L-SYSTEM AMINO ACID TRANSPORTER. T.J. Woodlock*, D.A. Young*, T.R. Boal*, M.A. Lichtman, and G.B.Segel*. Univ. of Rochester, NY 14642

Chronic lymphocytic leukemia (CLL) B cells have markedly diminished membrane L-system amino acid transport as compared with normal mature B and T lymphocytes. L-system functional recovery is induced in these cells by the maturational agent, tetradecanoyl phorbol acetate (TPA). The L-system functional recovery in CLL cells was exploited to identify possible candidate proteins for the L-system transporter. Membrane proteins of resting and TPA-matured CLL B cells were studied using giant 2-dimensional gel electrophoresis. Proteins were metabolically labeled with [^{35}S]methionine, and in separate experiments, proteins were photolabeled with [^{125}I]iodoazidophenylalanine which is transported by the L-system and which binds to the L-system carrier. The metabolic label identified 1400 membrane proteins, nearly all of which were present both in resting and in TPA-treated cells. Following TPA treatment, 14 new metabolically labeled maturation-associated membrane proteins were identified. The photolabel identified 450 membrane proteins both in resting and in TPA-treated cells. Five TPA-induced new proteins were co-identified by metabolic labeling and by photolabeling with the L-system photoprobe. Given these labeling characteristics, one or more of these 5 proteins may be related to the L-system carrier.

9.19

INCREASED ARGININE UPTAKE IN CULTURED SKELETAL MUSCLE CELLS AFTER TRANSFECTION WITH SYSTEM y^+ TRANSPORTER cDNA. L.B.M. Tadros*, E. Robertson*, P.M. Taylor* & M.J. Rennie. Dept of Anatomy & Physiology, University of Dundee DD1 4HN, Scotland UK.

Neonatal rat skeletal muscle cells in primary culture were transfected with cDNA for the cationic amino acid transport system y^+ in a eukaryotic expression vector containing the SV40 promoter (pJET clone; see Kim et al, *Nature* **352**: 725-728, 1991 for rationale). Transfection was performed using a calcium phosphate-DMSO technique with 5-10ug DNA/50µg cell protein. Both 5-day myoblast and 9-day myotube cultures showed increased uptake of 0.01mM [^{3}H]arginine 85h post-transfection compared to sham-transfected controls:-

	control	pJET transfected
myoblasts	79± 7	145± 6
myotubes	73±12	196±14

(all values in pmol/mg cell protein.min; means±sem for 3 transfections from a single culture). The results indicate that cultured muscle cells are capable of functional expression of transfected cDNA for membrane transporters. Such transfected cells may provide a useful model system for studying cellular regulation of amino acid transport in skeletal muscle.

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9.20

INDEPENDENT REGULATION OF ARGININE AND ALANINE TRANSPORTERS IN HUMAN INTESTINAL EPITHELIAL CACO-2 CELLS. Ming Pan, W.W. Souba, and Bruce R. Stevens. Dept. of Physiology, Univ. of Florida Coll. Medicine, Gainesville, FL 32610-0247.

Membrane transporters serving arginine (Na-independent system y+) and alanine or glutamine (Na-dependent system B) were investigated in Caco-2 cells. The Vmax of each transporter was an order of magnitude greater in undifferentiated cells (2 days old) compared to differentiated cells (9 days old), while each Km value was unaffected by cell differentiation status. In cell monolayers exposed to buffers that contained only single amino acids, it was found that amino acid substrates unique to system y+ stimulated the activity of system y+ only, while only substrates of system B stimulated cotransport via system B. For each transporter system, the ranking of amino acid stimulation was directly correlated with the degree of competitive analogue inhibition. Cycloheximide prevented the specific substrate-induced activation of either system y+ or system B during prolonged (>24 hr) exposure to the appropriate substrate. Peptide growth factors EGF and TGFα (>40 hours incubation) each increased system B activity by 100%, and their activation effect was abolished by cycloheximide. In contrast, cycloheximide did not affect the 50% activation of system y+ by EGF or TGFα. Phorbol ester (TPA, >8 hours incubation) stimulated system B activity by 100% in a cycloheximide-sensitive manner. Arginine uptake via system y+ was also stimulated by phorbol ester, but the effect was insensitive to cycloheximide. Chronic exposure to growth factors and phorbol ester was required to activate each transporter system, and only the Vmax was affected (not Km). The specific protein kinase C inhibitor, chelerythrine, abolished the TGFα/EGF and TPA stimulation of system B or system y+ activity. Together these data suggest that: (1) Caco-2 epithelial differentiation status is associated with regulation of amino acid transport; (2) amino acid transporter system B and system y+ are regulated independently; (3) transporter substrates up-regulate their own transporter activity via trans-stimulation or *de novo* protein synthesis of new membrane transporter proteins; (4) EGF and TGFα act through a protein kinase C pathway to up-regulate system B activity likely via *de novo* protein synthesis of membrane transporters, while system y+ activity may be mediated directly by protein kinase C.

9.21

ONTOGENY OF THE SYSTEM y^+ CATIONIC AMINO ACID TRANSPORTER IN RAT PLACENTA: CORRELATION OF ACTIVITY, PROTEIN, AND mRNA LEVELS. Marc S. Malandro*, Mark J. Beveridge*, Donald A Novak*, and Michael S. Kilberg. Univ. of Florida, Dept. of Biochem. and Mol. Biol. and Dept. of Peds., Gaivesville, FL 32610

Transport of amino acids from maternal to fetal circulation is essential for the proper development of the fetus. The cell type responsible for this transfer is the placental syncytiotrophoblast. To investigate the developmental regulation of placental transport activity in response to transcriptional and translational control, we have focused on the System y^+ amino acid transporter. This system is responsible for the sodium-independent cationic amino acid transport. [^{3}H]Arginine uptake by microvillous membrane vesicles prepared from rat placentas at 14 to 21 days gestation exhibits a decrease with development. When anti-System y^+ antibodies were used to perform immunoblot analysis of microvillous membrane proteins prepared from placentas of the same day gestation, the decrease in transport activity with increasing age was accompanied by a decrease in protein content as well. Northern analysis of RNA prepared from placentas of the same gestational age also showed a parallel decrease in the amount of System y^+ mRNA. These data demonstrate the normal decrease in cationic amino acid transport activity as pregnancy progresses is, at least in part, the result of a decrease in the mRNA and accompanying protein levels of the System y^+ carrier.

9.22

EXPRESSION OF THE mRNA FOR THE Na-DEPENDENT CATIONIC AMINO ACID TRANSPORTER IN THE RAT SMALL INTESTINE. Mônica Puppi*, R. Adam Noel* and Susan J. Henning* (SPON: S. G. Schultz). Baylor College of Medicine, Houston, TX 77030

EcoR, the membrane receptor for the ecotropic murine leukemia virus, functions as a cationic amino acid transporter. It shows saturable, stereo-specific, and sodium-independent uptake, findings consistent with the behavior of y^+, the principal mammalian cationic amino acid transport system for most cells in the body. In order to study the expression of EcoR in the rat intestine, we cloned the rat cDNA homolog of murine EcoR by screening a rat intestinal cDNA library with the murine EcoR cDNA (kindly supplied by Albritton *et al.*). A clone designated MP10 of approximately 7.9 kb was obtained and corresponded to the entire mRNA. Partial sequencing of MP10 revealed similarity higher than 80% to the mouse 5' end. A developmental Northern blot of total jejunal RNA probed with MP10 revealed two increases in the levels of EcoR mRNA. The first occurred at fetal day 20, and the second occurred at postnatal day 11. Levels then declined and remained low through adulthood. When investigating the expression of EcoR along the length of the intestine, we found the abundance of the mRNA to be similar in jejunum, ileum and colon, and slightly lower in the duodenum. To assess whether EcoR is expressed by the proliferating vs. the differentiated cells of the intestinal epithelium, we studied Caco-2 cells (which spontaneously differentiate in vitro). Higher levels of EcoR mRNA were seen in nondifferentiated cells, declining to low levels as the cells differentiate. Overall, the data suggest that y^+ does not account for reported changes of cationic transport during development and differentiation. This work was supported by NIH grant #HD-14094.

9.23

TAURINE TRANSPORT IN A HUMAN COLON CARCINOMA CELL LINE: EXPRESSION AND PROTEIN KINASE C - DEPENDENT REGULATION. Matthias Brandsch*, Yusei Miyamoto, Vadivel Ganapathy and Frederick H. Leibach. Medical College of Georgia, Augusta, GA 30912-2100

The human colon carcinoma cell line HT-29, grown as a confluent monolayer on an impermeable plastic support, accumulates taurine against a concentration gradient. The uptake is dependent on Na^+ and on Cl^-. The stoichiometry of taurine:Na^+:Cl^- is 1:2:1. Kinetic experiments strongly suggest that a common transporter is responsible for the uptake of taurine and ß-alanine. Phorbol 12-myristate 13-acetate (PMA), a phorbol ester known to stimulate protein kinase C, rapidly inhibits the taurine uptake by about 75 %. This inhibition proceeds in a dose-dependent manner with an IC_{50} value of 43 nM and is associated with a decrease in the maximal velocity and in the transport affinity. The effect of PMA can be blocked by co-treatment of the cells with staurosporine, an inhibitor of protein kinase C. Neither inhibitors of protein synthesis nor colchicine prevents the inhibitory effect of PMA. It is concluded that the activity of the taurine transporter in HT-29 cells is under regulatory control of protein kinase C, most likely via phosphorylation of the transporter proteins.

9.24

THYROXINE AND TRIIODOTHYRONINE ARE COMPETITIVE INHIBITORS OF PHENYLALANINE TRANSPORT INTO A MOUSE NEUROBLASTOMA CELL LINE, NB41A3. M. Lakshmanan, Department of Medicine, MetroHealth Medical Center, Cleveland, OH 44109.

Amino acids and thyroid hormones are transported into cells by stereospecific carrier mediated processes which exhibit Michaelis-Menten (M-M) kinetics. Thyroid hormone transport into a mouse neuroblastoma cell line, NB41A3, and into rat and human red blood cells is competitively inhibited by neutral aromatic amino acids. To further define the relationship between aromatic amino acid and thyroid hormone transport, the transport of phenylalanine (Phe) into NB41A3 cells was studied. The initial velocity of tritiated L-Phe uptake into confluent NB41A3 cells is linear for up to 2 minutes at room temperature for substrate concentrations between 1 nM and 10 uM. Therefore, 0.5, 1.0, 1.5, and 2.0 min uptakes in duplicate were used to determine the initial velocity of cell uptake at each of 12 concentrations by linear regression. The resulting data was fit to the M-M equation by Scopfit (Duke University). The M-M parameters for Phe transport are $K_m = 2.2 \pm 0.1$ uM and $V_{max} = 110 \pm 30$ pmol/min x 10^6 cells (mean$\pm$S.D). L-thyroxine (L-T_4) and L-triiodothyronine (L-T_3) are competitive inhibitors of Phe transport with a $K_i = 2.4$ uM and $K_i = 1.5$ uM, respectively. D-analogues of Phe, T_4, T_3, or tryptophan at 1 uM have no effect on L-Phe uptake while N-ethylmaleimide is an irreversible non-competitive inhibitor of transport. A member of a novel class of thyromimetics, 3,5,-dibromo-3'-pyridazinone-L-thyronine also inhibits L-Phe transport. Even though physiological concentrations of thyroid hormones do not interfere with Phe transport, the effect seen in this study is further evidence for an association between thyroid hormone and aromatic amino acid transport across the plasma membrane.

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9.25

VALINE INDUCES NA^+ CURRENT IN MONOLAYERS OF SALAMANDER SMALL INTESTINAL EPITHELIAL CELLS DERIVED FROM PRIMARY CULTURE OF CELL NESTS. John F. White, Department of Physiology, Emory University, Atlanta, GA 30322.

Cell nests, subepithelial clusters of germinative stem cells, were isolated from the small intestine of Amphiuma by digestion with or without the use of collagenase (1mg/ml). When incubated in a culture medium the nests attached to collagen-coated culture dishes and spread to form subconfluent monolayers. Cells were viable in culture for up to 3 weeks. To assess whether the cultured cells possess Na-valine cotransport, a characteristic of mature small intestinal cells, whole cell current was measured using the patch clamp technique. In 19 of 26 cells L-valine (25 mM) produced an inward current reversible upon washout and averaging 25.3 ± 6.6 pA. Valine also depolarized the cell by an average of 14.5 ± 1.6 mV. The magnitude of the current was a saturable function of the bath valine concentration; from Lineweaver-Burk analysis the K_m = 14.8 mM. The response to valine was eliminated when medium Na was replaced mole for mole with Tris. L-alanine also induced an inward current whereas D-valine did not. The results suggest that cells derived from the nests are capable of Na-valine cotransport. Monolayers derived from nests provide the first primary culture of small intestinal cells exhibiting Na-amino acid cotransport.

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9.26

TUMOR NECROSIS FACTOR (TNF) STIMULATES SODIUM-DEPENDENT AMINO ACID TRANSPORT IN HEPATOCYTE PLASMA MEMBRANE VESICLES. Wiley W Souba and Anthony J Pacitti*, Dept of Surgery, University of Florida, Gainesville, FL 32610.

Severe infection is characterized by a translocation of amino acids from the periphery to the liver, an event which appears to be mediated, in part, by the cytokine tumor necrosis factor-alpha (TNF). However, little is known about the *in vivo* effects of TNF on the various plasma membrane transport systems catalyzing hepatic amino acid uptake. We investigated the activity of Na-dependent transport Systems A, ASC, and N and the Na-independent transport of leucine and glutamine in hepatic plasma membrane vesicles (HPMVs) prepared from rats treated with TNF. HPMVs were prepared by differential centrifugation. Vesicle purity was verified by electron microscopy and enzyme marker enrichments. Treatment of animals with TNF resulted in time- and dose-dependent 500% and 50% maximal increases in System A and System N activity, respectively, secondary to an increase in transport Vmax. Maximal increases in transport were observed 4 hrs after exposure to TNF and had returned to basal levels within 24 hours. Similarly, System ASC activity was stimulated 80% in HPMVs prepared from rats receiving TNF. In contrast, the Na-independent transport of leucine and glutamine was unaffected by prior TNF treatment. The marked enhancement of Na-dependent amino acid transport activity by TNF represents an important mechanism underlying the accelerated hepatic amino acid uptake that occurs during critical illness.

9.27

PROPERTIES OF GLY-SAR UPTAKE IN HUMAN COLON CARCINOMA CELL LINES, CACO-2 AND HT-29. Yusei Miyamoto*, Matthias Brandsch*, Vadivel Ganapathy and Frederick H. Leibach. Medical College of Georgia, Augusta, GA 30912-2100

Gly-Sar uptake was characterized in confluent monolayers of Caco-2 and HT-29. An inwardly directed H^+-gradient stimulated Gly-Sar uptake in Caco-2. On the other hand, Gly-Sar uptake in HT-29 was unresponsive to the H^+-gradient. The absence of a H^+-gradient-dependent Gly-Sar uptake in HT-29 was evident whether the cells were cultured in the presence of glucose or galactose. The uptake of Gly-Sar measured in the presence of a H^+-gradient was 20 times less in HT-29 than in Caco-2. Gly-Sar uptake was markedly inhibited by di- and tripeptides, but not by amino acids, in Caco-2. Acidification of intracellular pH in Caco-2 by NH_4Cl prepulse decreased Gly-Sar uptake. The K_t and V_{max} values for the uptake process in Caco-2 were 1.14 ± 0.07 mM and 17.7 ± 0.6 nmol/ mg of protein per 10 min, respectively. It is concluded that the peptide-H^+ cotransporter, which exists in normal small intestine, is expressed in Caco-2 but not in HT-29 cells. (Supported by NIH grant DK 28389.)

9.28

EVIDENCE FOR THE COUPLING OF CHLORIDE AND ALANINE UPTAKES IN SEAWATER EEL INTESTINAL BRUSH BORDER MEMBRANE VESICLES. A. Corcelli, A. De Giorgi, C. Ferri, Istituto di Fisiologia Generale. Universita' di Bari. Via Amendola 165/a. 70125 Bari. Italy.

The absorption of water and salt in seawater euryhaline fish intestine is driven by the transepithelial transport of Cl^-. We have examined the possibility of coupling between alanine and Cl^- fluxes across the brush border membrane of seawater eel entherocytes. Seawater eel intestinal brush border membrane vesicles were isolated by the Mg^{++} precipitation method and $^{36}Cl^-$ uptake measured by rapid filtration. Cl^- uptake was stimulated (up to 50%) in the presence of both L-alanine and sodium ions in the extravesicular medium. Extravesicular alanine had no effect in the absence of sodium ions. When the alanine gradient was abolished, i.e. in the presence of equilibrated alanine and Na^+ gradient, the stimulating effect of the amino acid was strongly reduced. Intravesicular L-alanine, in the presence of an inwardly directed sodium gradient, did not stimulate chloride uptake. Furthermore Cl^- uptake could be driven against a concentration gradient by the presence of both inwardly directed L-alanine and sodium gradients. The ability of L-alanine to stimulate chloride uptake was partially shared by D-alanine, but not by L-glutamate, L-proline, taurine, ß-alanine or D-glucose. The Na^+ and alanine dependent Cl^- uptake was specifically inhibited by 0.1mM harmaline or harmine. These drugs had no effect on Cl^- uptake in the absence of extravesicular alanine. Our data suggest the presence of a Na^+/alanine/Cl^- cotransport system in seawater eel intestinal brush border membrane vesicles; such a cotransport system could have a specific role in the osmotic homeostasis of euryhaline fish.

9.29

ROLE OF ANIONS IN CHOLINE UPTAKE BY MEMBRANE VESICLES OF LUNG A549 CELLS. Arnost Kleinzeller, Chandra Dodia*, Avinash Chander and Aron B. Fisher. Institute for Environmental Medicine, University of Pennsylvania, Philadelphia, PA 19104.

Alveolar type II pneumocytes and A549 cells take up choline (5-100 μM) against sizable concentration gradients by separate, Na^+-dependent and Na^+-independent pathways. These processes were now studied using randomly oriented plasma membrane vesicles from A549 cells. Choline binding by the preparation did not exceed 70 pmol/mg protein; the uptake of free choline (5 μM) proceeded against gradients of up to 50 fold. The overshoot for the Na^+-dependent transport at 10 min was dependent on the Na^+ gradient [out>in], but differed in time scale from that for amino-isobutyrate (AIB) (overshoot at ~2 min) when Cl^- was the anion present. The involvement of an additional gradient responsible for the delay of the overshoot was verified: lipid-soluble anions (HCO_3^-, acetate, SCN^-) reduced the overshoot time close to that for AIB, while hydrophilic anions (P_i, methyl sulfate) prolonged or abolished it. The up-hill choline uptake was abolished by the presence of protonophore (FCCP) plus valinomycin. The data are consistent with the suggestion that $choline^+$ and Na^+ are co-transported into vesicles by an electrogenic process requiring permeable anions for charge neutralization. The Na^+-independent transport pathway appears to be an electrogenic $choline^+$-H^+ countertransport (amiloride-sensitive) system, with anions again determining the rate of charge dissipation.

9.30

ISOLATION OF A PLASMA MEMBRANE CHOLINE BINDING PROTEIN FROM A LUNG TUMOR CELL (A549). Un-Jin P. Zimmerman, Celeste H. Campbell, Brian B. Hennigan and Aron B. Fisher. Inst. for Environ. Med., Univ. of Pennsyl., Phila., PA 19104.

Isolation of the choline transport protein from lung epithelial cells has been initiated using a bronchoalveolar carcinoma cell line (A549). Plasma membrane was purified from the cultured A549 cells by sucrose density gradient centrifugation. The plasma membrane was then solubilized in 2% sodium deoxycholate and subjected to affinity chromatography using trimethyl-p-aminophenyl ammonium conjugated to Sepharose 4B equilibrated in glycylglycine buffer (pH 7.4) containing 200 mM NaCl and 0.01% deoxycholate. Affinity bound proteins were eluted from the column with buffer containing excess choline. The eluted fractions were vacuum concentrated, exhaustively dialyzed and assayed for [^{3}H]-choline binding activity. The affinity purified fraction representing about 0.1% of the initial protein, has 10^3-fold enrichment in the specific choline binding activity relative to the initial plasma membrane preparation. Analysis by SDS PAGE revealed a single polypeptide band with apparent molecular mass of 38 kDa. The choline binding activity of this fraction was abolished when heat denatured and was inhibited by n-decyl choline and dimethyl ethanolamine but not by hemicholinium-3. The results indicated isolation of a plasma membrane choline binding protein that may represent the low affinity choline transport protein.

9.31

DEVELOPMENT OF A PHOTOAFFINITY PROBE FOR CARDIAC ADENOSINE BINDING PROTEIN. Michael J. Rovetto, Julie A. Zimmerschied, Tony Haynes, and Calvin C. Hale. Univ. of Missouri, Columbia, MO 65211.

The absence of a high specific activity, high energy radioisotopic probe for nucleoside binding proteins has hampered isolation and identification of adenosine transport protein. One probe which has been available is nitrobenzylthioinosine (NBMPR) but this has been labeled only with ^{3}H. This coupled with low efficiency of covalent association with proteins makes NBMPR a poor probe. Therefore we undertook to develop a probe labeled with ^{125}I. To do this we reacted N-hydoxysuccinimidyl-4-azidosalicylicacid (NHS-ASA) with 5'-amino-5'-deoxyadenosine. This reaction resulted in 4 major peaks on C18 reverse phase HPLC. After iodination, these peaks were used to probe purified cardiac sarcolemmal vesicles with adenosine transport activity. Three of the peaks labeled proteins upon photoactivation (exposure to uv light). One of these peaks labeled a single protein with an apparent molecular weight of 70 kD. The labeling was specific for adenosine binding as adenosine reduced photolabeling. Further analysis by 2-D gel electrophoresis yielded a single radiolabeled protein with an isoelectric point of about 6.3. Supported in part by NIH R01 HL27336 (MR) and AHA-MO Affiliate (CCH).

9.32

RIBOFLAVIN UPTAKE BY MICROVILLOUS AND BASAL MEMBRANE VESICLES FROM HUMAN PLACENTAL SYNCYTIOTROPHOBLAST. Aaron J. Moe, David R. Plas*, Kristen A. Powell*, and Carl H. Smith. Department of Pediatrics, Washington University School of Medicine, Children's Hospital, St. Louis, MO 63110.

In order to understand the mechanism for transplacental riboflavin flux we have characterized [^{3}H]riboflavin uptake by membrane vesicles isolated from the apical (maternal-facing) and basal (fetal-facing) membranes of the syncytiotrophoblast from full-term human placentas. [^{3}H]riboflavin uptake increased with incubation time in both membranes and reached equilibrium by 60 min. Equilibrium [^{3}H]riboflavin uptake was completely insensitive to variations in incubation medium osmolality in contrast to [^{3}H]alanine which was into an osmotically sensitive space. Osmotic insensitivity suggests riboflavin binding to a membrane component. Riboflavin binding was characterized by incubating vesicles with increasing amounts of unlabeled riboflavin and determining equilibrium uptake. In microvillous membrane vesicles binding constants were: K_d = 0.99±0.36 μM, B_{max}= 1.0±0.34 pmol/mg protein, and coefficient of non-specific binding 5.9 x 10^{-7}. The corresponding values in basal membrane vesicles were 0.52±0.45 μM, 0.2±0.1 pmol/mg protein, and 6 x 10^{-7}. It remains to be resolved whether there is a separate transport mechanism or how the specific binding participates in transplacental transport.

9.33

ACINAR HETEROGENEITY IN HEPATIC MEMBRANE TRANSPORT. RH Moseley, S Jarose*, and P Permoad*. VA Medical Center and University of Michigan School of Medicine, Ann Arbor, MI

Liver perfusion studies suggest that the transport of α-ketoglutarate (KG) and glutamate (GLU) exhibit acinar heterogeneity, in that the uptake and metabolism of these organic anions appears to occur predominantly in the perivenous region of the hepatic acinus. However, this model cannot distinguish differences in either the rates of solute uptake and/or efflux or intracellular binding and/or metabolism. The acinar localization of KG and GLU transport was, therefore, examined using rat basolateral liver plasma membrane vesicles (blLPMV) isolated from control animals and animals treated 24 h before with selective perivenous and periportal toxins (CCl_4 (3.9 mmol/kg), and allyl alcohol (AA; 0.62 mmol/kg), respectively). In control blLPMV, KG and GLU uptake exhibited features as previously described. In blLPMV from CCl_4-treated animals, no Na^+ gradient-driven KG uptake could be demonstrated although Na^+ gradient-driven [^{3}H]taurocholate (TC) uptake was preserved. In blLPMV from AA-treated animals, both Na^+ gradient-driven KG and TC uptake were preserved. Recovery of Na^+ gradient-driven KG transport was observed in blLPMV derived from animals treated 7 days before with CCl_4. In contrast, there was no difference in GLU uptake, which was largely Na^+-independent, in blLPMV from control, AA-, and CCl_4-treated animals. These studies functionally confirm the heterogenous localization within the hepatic acinus of basolateral Na^+-dependent dicarboxylate, but not GLU, cotransport. Work underway aimed at cloning this transport protein may provide a tool with which to address the mechanisms responsible for the selective expression of basolateral Na^+-dependent α-KG transport in the perivenous region of the hepatic acinus.

10.1

REGULATORY VOLUME DECREASE (RVD) IN APLYSIA NEURONES. Eliana Scemes* and Antonio C. Cassola* (SPON: G. Malnic) IB and ICB, Univ. de São Paulo, São Paulo, SP, 05508, Brazil.

Exposure of Aplysia neurones to hypotonic solutions leads to a initial rise in cell volume, followed by a regulatory volume decrease. The initial rise is critically dependent on external $[K^+]$: if external $[K^+]$ is kept at control value the initial volume increase approaches the expected for a osmometric behavior. Proportional reduction of external $[K^+]$ limits the initial swelling indicating that, under this condition, early solute loss reduces cell osmolality. The regulatory volume decrease restores the initial cell volume in about 10 minutes, after hypotonic shock. $BaCl_2$ (10mM) slow down the process of volume recovery, without blocking it. Furosemide, in the micromolar range, reversibly impairs the volume recovery. The results favor the hypothesis that conductive pathway and furosemide-sensitive pathway, probably the Na-2Cl-K cotransport, are the main route for solute loss and cell volume recovery in these invertebrate cells. (FAPESP Proc. 90/2154-1)

10.2

CHARACTERIZATION OF REGULATORY VOLUME DECREASE (RVD) IN THE HUMAN MYELOID THP-1 AND HL6^ CELL LINES. Elaine K. Gallin and Toni M. Mason*, Physiol. Dept., Armed Forces Radiobiology Research Inst. Bethesda, Md 20889

Exposure to hypotonic (.59X) Hanks' produces a transient increase (145%) in volume followed by RVD in both THP-1 and HL60 cells. Incubating cells in .59X high K/low Na Hanks' blocked RVD, suggesting that K efflux was required. The K channel blockers quinine (0.5-4mM) and barium (2-4mM) blocked RVD in both cell types, while addition of 4-aminopyridine (5mM) and charybdotoxin (CTX;25 nM) had no affect. Gramacidin (1uM) induced volume changes when added to cells in .59X Hanks' containing quinine, indicating that quinine inhibited RVD by blocking a cation permeability and that the anion permeability of both cell lines was high. However, gramacidin produced little or no volume change in cells in 1X Hanks', suggesting that the basal Cl permeability of both THP-1 and HL60 cells was low. 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS;0.5mM) did not block RVD in either cell line. In HL60 cells, .59X high K Hanks' produced a secondary swelling. In contrast, .59X K Hanks' induced a secondary swelling in THP-1 cells only after addition of gramacidin, suggesting that the cation permeability of hypotonically stressed THP-1 cells is lower than in similarly stressed HL60 cells. RVD occurred in 0 Ca-EGTA (1mM) Hanks' in both cell lines. Our data indicate that while some differences exist in the volume regulatory responses of these two myeloid cell lines, both a SITS-insensitive anion permeability and a quinine/barium-inhibitable, CTX-insensitive K permeability are activated by swelling in THP-1 and HL60 cells.

10.3

HYPOTONIC ACTIVATION OF Na^+/H^+ ANTIPORT IS REQUIRED FOR SMALL VOLUME RVD IN VILLUS EPITHELIAL CELLS. R. John MacLeod*, P. Lembessis* and J.R. Hamilton*. (SPON: H. Tenenhouse). Dept. of Paediatrics, McGill University, Montreal, Canada

To further characterize the signal transduction of the K+ conductance activated for Regulatory Volume Decrease (RVD) in villus epithelial cells isolated in suspension from guinea pig jejunum, we measured changes in intracellular pH (pH_i) by fluorescent spectroscopy and monitored cell volume with electronic cell sizing. Small hypotonic dilutions, 5% or 7%, caused cell swelling followed by RVD which were prevented ($p<.001$) by 1 μM N-methyl isobutylamiloride (MIA) an inhibitor of Na/H antiport. RVD was also prevented in Na^+-free medium (final relative volume 1.04 ± .01, $p<.001$). RVD after 20% or 30% hypotonic dilutions were not effected by MIA or Na^+-free medium. After 5% hypotonic dilution, pH_i alkalinized which was prevented by 1 μM MIA (ΔpH_i 0.065 ± 0.011 vs 0.016 ± 0.007, $p<.005$, n=6). Charybdotoxin (CTX, 50nM) an inhibitor of Ca^{2+}-activated K^+ conductance prevented RVD after 5% hypotonic dilution (1.04 ± .01, $p<.001$) but had no effect on RVD after 20% hypotonic dilution. Therefore, in K^+ rich medium, villus cells were diluted 50% then pulsed with 50mM NH_4Cl. This caused a transient alkalinization (ΔpH_i=0.390) and a sustained 2° swelling (1.16 ± .01 vs 1.25 ± .01, $p<.05$) which was prevented by CTX (1.19 ± .02, $p<.05$). We conclude that small volume increases, which mimic Na^+-nutrient absorption, activate basolateral Na/H antiport. Resulting alkalinization is required for CTX sensitive K^+ conductance. In contrast with symmetrical cells, Na/H antiport may be activated hypotonically in Na^+ absorbing epithelial cells.

10.4

Sodium Efflux in Dog Erythrocytes, Earl Dixon and Denise Spencer, School of Veterinary Medicine, Tuskegee University, AL 36088

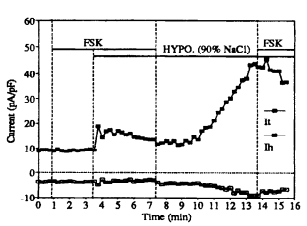
Recent studies suggested that sodium movements across the dog erythrocyte membrane in response to volume regulation occur via Na/H exchange. Studies in our laboratory were designed to quantitate sodium movements using rapid-sampling techniques. Sodium efflux was quantitated by sampling the media bathing isotopically Na-loaded cells at 3 minute intervals. Sodium efflux was quantitated under isotonic and hypertonic conditions. Several inhibitors (Amiloride, Ethylisopropylamiloride(EIPA), Dimethyl-amiloride(MPA), Quinidine, Clonidine, Harmaline and Cimetidine) were observed to inhibit sodium transport. Experiments designed to study sodium efflux following an abrupt increase in osmolarity from 300 to 400 mOsm showed that there was an immediate increase in sodium efflux. The results of these studies confirmed that the major transport component in shrinkage-activated sodium efflux involved Na/H exchange and sodium movement was affected by a series of drugs that have been showed to inhibit this pathway. These results further confirm the involvement of Na-H exchange in volume regulatory processes.

(Supported in part by Grants; NSF DCB-9005301, NIH 5 K14 HL01994, and NIH S06 GM0891).

10.5

CYCLIC AMP INHIBITS THE SWELLING ACTIVATED I_{Cl} ASSOCIATED WITH *CARDIAC CELL* VOLUME REGULATION J. Zhang*, T.W. Smith*, L.A. Lobaugh*, S.K. Hall*, and M. Lieberman. Department of Cell Biology, Division of Physiology, Duke University Medical Center, Durham, NC 27710

Cultured chick heart cells swollen in hypoosmotic solutions rapidly (<3 min) generate an outwardly rectifying Cl^- current (holding potential, -40 mV; test potential, +60 mV) as well as a regulatory volume decrease (RVD). Hypoosmotically induced cell swelling (10 min) is also associated with a decrease of cell cAMP content to 72±2% (n=3). Forskolin (10 μM), an activator of adenylyl cyclase, reversibly elevates cAMP in both isosmotic and hypoosmotic solutions, and inhibits the RVD. The swelling activated Cl^- current is depressed by the addition of forskolin before *or* after the change to hypoosmotic solution (see Figure). Isoproterenol (20 μM), a β-adrenergic agonist that elevates cAMP, also inhibits the swelling activated I_{Cl}. These data indicate that cAMP acts as an intracellular signal transducer to modulate a sarcolemmal transport pathway involved in cardiac cell volume regulation. *Supported in part by NIH HL27105; TWS is an AHA Med. Student Research Fellow.*



10.6

REGULATION OF CELL VOLUME AND PH BY ION CHANNELS: A COMPUTER ANALYSIS. Harold G. Hempling, Department of Physiology, Medical University of South Carolina, Charleston, S.C. 29425.

Programs and subroutines have been written for the Macintosh computer which 1. model changes in cell volume and pH in response to a weak acid and/or a weak base 2. estimate buffering power from changes in cell volume 3. estimate permeability of the chloride/bicarbonate exchanger 4. predict the effect of the K^+/H^+ and the Na^+/H^+ exchangers on cell volume and pH 5. predict the behavior of a cotransporter with neutral and electrogenic properties. Programs were validated from experiments on erythrocytes and from published data in the literature. Supported in part by the American Heart Association, S.C. Affiliate.

10.7

GENETIC CONTROL OF RED CELL VOLUME REGULATION. Pearl R. Fernandes and Michael J. Dewey (SPON: M.B. Wolf). Univ. of South Carolina, Columbia, SC 29208.

There are few genetic variants useful for studies of cellular volume regulation. In mice we have recently defined a gene, rol, which is involved in controlling cellular water and ion contents. Rol was originally defined for its effects on osmotic fragility; red cells from mice with the resistance (rol^r) allele manifest extreme resistance to osmotic lysis, whereas the sensitive (rol^s) allele imparts normal osmotic fragility. For detailed physiological characterization of the gene, we have developed congenic strains of mice differing only at rol. We have found that, relative to rol^s red cells, rol^r ones have less cell water, 20 mM less K, a reduced MCV, and an elevated MCHC. Furthermore, from ^{86}Rb influx studies, the sodium pump activity is 30% elevated in rol^r cells, and transport through the [Na,K,Cl] pathway is 2- to 4-fold elevated. On the other hand rol does not affect the amount of hemoglobin/cell and is not the structural gene for the alpha or beta chains of hemoglobin. Differences in levels of ATP or 2,3 DPG do not account for the effects of rol. Analyses of membrane proteins, their phosphorylation and palmitoylation, and the cellular lipid composition (fatty acids and phospholipids) did not reveal any structural differences. Rol could be the structural gene for one of the transport proteins or some regulatory element involved in the control of transport activity. It will be of interest to study the interaction of rol with genes such as beta-thalassemia or sickle cell anemia in the final outcome of their pathologies.

MEMBRANE ATPASES AND OTHER ION TRANSPORT SYSTEMS

11.1

VOLTAGE-DEPENDENT BINDING OF K^+ TO THE Na^+/K^+ PUMP IN SINGLE RABBIT CARDIAC PURKINJE CELLS. Fons Verdonck, Frieda V.Bielen*, Helfried G.Glitsch*. Univ.Leuven Campus Kortrijk, 8500 Kortrijk, Belgium; Ruhr Univ., Bochum, FRG

The aim of the present study was to investigate the voltage (V)-dependence of the Na^+/K^+ pump current (I_p) as a function of the external K^+ concentration ($[K^+]_o$). The cells were superfused with Na^+-free media shifting the rate-limiting step to the K^+ translocating part of the pump cycle. At zero V, half maximal I_p activation decreased from 1.9 mM $[K^+]_o$ in Na^+-containing to 0.08 mM ($K_{0.5}$) in Na^+-free medium. At $[K^+]_o$ above $K_{0.5}$ the I_p-V curve displayed a positive slope between -100 and -20 mV and a plateau at more positive potentials. At lower $[K^+]_o$, I_p increased at negative V and the I_p-V curve showed a negative slope. The apparent affinity of the pump for K^+ is V-dependent and $K_{0.5}$ versus V can be described by a single exponential function. The factor α determining the steepness of the function amounts to 0.24. These findings are consistent with the hypothesis of a binding site within the sarcolemma accessible via an channel-like structure, although V-dependent changes in the conformation of the binding site causing variation in the K^+ affinity cannot be excluded.

11.2

RAT KIDNEY NA/K PUMPS FUSED INTO IMMATURE LK SHEEP RED CELLS INTERACT WITH THE L_p ANTIGEN AND ACQUIRE THE DISTINCTIVE BEHAVIOR OF MATURE LK PUMPS FOLLOWING MATURATION IN VITRO. Z.-C. Xu[1]*, P.B. Dunham[2] and R. Blostein[1], [1]McGill University, Montreal, Canada and [2]Syracuse University, Syracuse, N.Y.

A genetic dimorphism of sheep red cells characterized by differences in K_{in} concentrations in mature low-K^+ (LK) and high-K^+ (HK) red cells reflects differences in their Na/K pumps and is linked to the ML blood group system. The L_p antigen is restricted to LK cells and anti-L_p antibody stimulates the Na/K pumps in these cells, mainly by relieving inhibition of LK pumps by L_p. We have observed that L_p is a molecular entity distinct from the pump; it can interact with pumps of exogenous origin, namely rat kidney Na/K pumps incorporated by fusion of microsomes into LK cells. Exogenous rat kidney and endogenous red cell pumps were distinguished by their different sensitivities to ouabain. Anti-L_p stimulated by >50% kidney pumps incorporated into immature LK sheep cells. This indicates that L_p is a distinct molecular entity free to inhibit exogenous pumps. Anti-L_p did not stimulate kidney pumps incorporated into mature LK cells, probably reflecting restriction of lateral movement of pumps and antigens by the cytoskeleton in mature cells, but did stimulate kidney pumps following in vitro maturation of microsome-fused LK reticulocytes. Exogenous kidney pumps, like endogenous LK reticulocyte pumps, are kinetically like pumps of HK cells. Following in vitro maturation, they acquired the distinctive kinetic characteristics of mature LK cells, i.e. they become sensitive to inhibition by K_{in}. Anti-L_p prevents the maturation-associated change in endogenous (red cell) as well as exogenous (kidney) pump kinetics. Trypsin, which destroys L_p antigen, also prevents maturation of the pumps. From these results it is concluded that Lp-Na/K pump interactions are the basis for the development of the distinctive behavior of Na/K pumps of mature LK cells. (Supported by MRC & QHSF (Canada) and NIH.)

11.3

NON-INVASIVE, REAL-TIME MONITORING OF NA/K ATPASE ACTIVITY VIA MICROPHYSIOMETRY. Donald L. Miller, John C. Olson, and John C. Owicki. (SPON: Martin Frank) Molecular Devices Corp., Menlo Park, CA 94025

The maintenance of the Na and K gradients across cell membranes consumes a fair amount of cellular energy production, and as such should be reflected in the cellular metabolic rate. We have found that the rate at which TE671 and CHO cells acidified their milieu within a Cytosensor microphysiometer was dependent on the Na pump activity of those cells. Addition of 500 µM ouabain or removal of K caused a decrease of about 10% of this acidification rate. Reapplication of K caused a transient overshoot in the rate which was inhibited in the presence of ouabain, and the amplitude of which was proportional to the time during which K was absent from the salts. We think the overshoot represents increased pump activity occurring as a result of $[Na]_i$ increases during the period the pump was off. The kinetics of the return of rate to baseline were exponential, and quite similar to the kinetics of $[Na]_i$ return as measured with a ratiometric fluorescence technique in the same cells. Increasing membrane Na permeability with ionophores caused a faster increase in $[Na]_i$ when K was absent and an increase in the amplitude of the overshoot. The pump contribution to the steady-state rate was independent of whether the cells were metabolizing in a glycolytic or oxidative mode, indicating both modes contribute to the energy demands of the pump.

11.4

ALDOSTERONE INCREASES THE Na^+,K^+-ATPase TOTAL AND CELL-SURFACE POOLS IN A6 CELLS. F. Verrey* and J. Beron* (SPON: H. Murer) Institute of Physiology, University of Zürich, CH-8057 Zürich.

Aldosterone induces in A6 cells a rapid increase in Na^+,K^+-ATPase α1 and ß1 gene transcription which is followed, ~3 hours after hormone addition, by an accumulation of the corresponding mRNAs and a 2-3 fold increase in the rate of subunit synthesis. Based on the observation that aldosterone induces an early increase in ouabain-binding sites which precedes this effect on the pump synthesis, it has been postulated that pumps could be translocated during the early response from an intracellular location to the cell surface (Pellanda et al., Am. J. Physiol. 262: C899-C906, 1992). To test this hypothesis we have used recloned A6 cells maintained on filter cultures in serum-free medium and selected for their responsiveness to aldosterone. Transepithelial Na^+ reabsorption was measured as short circuit current and the amount of total Na^+,K^+-ATPase by Western blotting with subunit-specific antibodies. To evaluate the relative size of the cell-surface Na^+,K^+-ATPase pool, cell-surface proteins were labeled from the apical or the basolateral side using sulfosuccinimidobiotin. After immunoprecipitation cell-surface Na^+,K^+-ATPase was revealed by blotting with streptavidin. Since the α subunit was not efficiently labeled, only the surface-expression of the ß subunit could be evaluated. Depending on the labeling procedure, 90%-100% of the signal for cell-surface ß subunit was basolateral. Five hours after aldosterone (10^{-6} M) addition Na^+ reabsorption was ~4 times higher than in control cells, while there was only a small increase in both total and cell-surface Na^+,K^+-ATPase. After 20 hours and 5 days Na^+ reabsorption was 11 and 15 times higher than the control values and the total Na^+,K^+-ATPase had increased by 100% and 150%, respectively. The level of cell-surface ß subunit paralleled these changes. We conclude that the late response to aldosterone is characterized by an increase in total and cell-surface Na^+,K^+-ATPase, while there is no indication that aldosterone promotes the translocation of pumps to the cell-surface. These results indicate that aldosterone might activate during the early response period previously silent cell-surface pumps.

11.5

PARATHYROID HORMONE (PTH) INHIBITS Na+,K+-ATPase ACTIVITY THROUGH A CYTOCHROME P450 PATHWAY. *Carla Ribeiro[1], *John Falck[2], *Michal Schwartzman[3] and Lazaro Mandel[1]. [1]Duke University, Durham, NC 27710; [2]University of Texas Heart Science Center, Dallas, TX 75235 and [3]New York Medical College, Valhalla, NY 10595.

PTH inhibits proximal tubule (PT) Na^+,K^+-ATPase activity independently of cAMP (Am. J. Physiol. 262: F209,1992). The signalling pathway responsible for this hormonal action was investigated in PT suspensions. 10^{-7}M PTH(1-34) did not affect cytosolic free Ca^{2+}, $(Ca^{+2})_i$, whereas 10^{-8}M angiotensin II (AII) increased it; since we showed that AII abolishes PTH-inhibited Na^+,K^+-ATPase, this PTH action is independent of alterations in $(Ca^{2+})_i$. Activation of guanylate cyclase is not involved either: 10^{-12}-10^{-6}M PTH(1-34) did not change cGMP levels. We next tested the effect of arachidonic acid (AA) on PTH-inhibited Na^+,K^+-ATPase activity. The analog PTH(3-34), which decreases pump activity by 25% without changing cAMP or $(Ca^{2+})_i$, was used. 10^{-6}M AA mimicked 10^{-8}M PTH(3-34)-decreased activity and, together, were not additive. 3 uM eicosatetraynoic acid (ETYA), an inhibitor of AA metabolism, blocked the PTH action. 10 uM indomethacin, an inhibitor of AA-dependent cyclooxygenase, did not prevent the PTH action, but 2 uM 7-ethoxy-resorufin, a cytochrome P450 inhibitor, prevented the PTH effect. Since PTH activates PT cytochrome P450 and increases 20-hydroxy-eicosatetraenoic acid, 20-HETE (Am. J. Physiol. 262:F591, 1992), the effect of 20-HETE was tested on pump activity. 10 uM 20-HETE inhibited Na^+,K^+-ATPase activity to the same extent as 10^{-8}M PTH(3-34) and was not additive with PTH. We conclude that PTH inhibits PT Na^+,K^+-ATPase activity by activating an AA-dependent cytochrome P450 pathway responsible for the generation of 20-HETE, a metabolite with natriuretic properties.

11.6

INVOLVEMENT OF ARACHIDONIC ACID METABOLITES IN REGULATION OF THE Na,K-ATPase IN MDCK CELLS. A. Moran, G. Rimon* and R. Cohen-Luria*. Departments of Physiology and Pharmacology, Faculty of Health Sciences, Ben-Gurion university of the Negev, Beer-Sheva, Israel.

It is well established that prostaglandins of the E series induce diuresis and natriuresis. We have recently shown that picomolar concentrations of PGE_2, but not PGE_1 or $PGF_2\alpha$, inhibits the activity of Na,K-ATPase and reduces the binding of ouabain, in a clone of MDCK cells. In the present study the possible involvement of arachidonic acid metabolites in the regulation of the Na,K-ATPase was tested. Treatment of the cells with 2 μM of aspirin, indomethacin or ibuprofen, blocked the PGE_2 induced inhibition of ouabain binding. In addition, the inhibitory effect of PGE_2 on Na,K-ATPase activity, also observed in Rb^+ flux, was abolished in the presence of ibuprofen. These results suggest the involvement of a cyclooxygenase pathway in the regulation of the Na,K-ATPase by PGE_2.

11.7

DIFFERENTIAL REGULATION OF TWO Na,K-ATPase β SUBUNIT mRNA SPECIES BY HYPERTONICITY IN AN MDCK CLONAL CELL LINE. Jesse W. Bowen and David W. Bollinger. Univ. of Missouri, Columbia, MO 65212

When MDCK cells (clone T) are incubated in hypertonic medium, Na,K-ATPase expression is stimulated, resulting in coordinate increases in α and β subunit mRNA (Am.J.Physiol. 262:C845, 1992). An MDCK subclone, S1, was isolated in our laboratory by limiting dilution of a heterogeneous MDCK culture obtained from the American Type Culture Collection. This subclone shared many characteristics with previously studied cells, including stimulation of α subunit Na,K-ATPase mRNA by hypertonicity. However, Northern blot analysis with a dog kidney β subunit cDNA probe, using high-stringency hybridization (55°C washes in 0.2X SSC, 0.2% SDS), revealed two mRNA species that were 2.65 Kb and 2.25 Kb in length. When MDCK/S1 cells were incubated in 500 mosmol/kg medium made hypertonic by adding NaCl, the concentration of the 2.65 Kb species increased significantly by 2.35 ± 0.24 (SE) times control ($p<0.01$, n=9) while the smaller mRNA increased only slightly and not significantly. Similar results were obtained when N-methyl-D-glucamine was used to increase the medium osmolality while holding the extracellular $[Na^+]$ at either 80 or 30 mM. Under these conditions, intracellular $[Na^+]$, thought to play an important role in the regulation of Na,K-ATPase expression, still rises, but to smaller extents than when NaCl is the added osmolyte. These results suggest either that this kidney cell line expresses multiple β subunit mRNA species that are differentially regulated, or that hypertonic cell shrinkage affects β subunit mRNA processing, resulting in different sizes of mature β mRNA from the same gene. (Supported by NIH GM40568)

11.8

THYROID HORMONE REGULATES NA,K-ATPASE α2 AND β2 IN SKELETAL MUSCLE. Kay K. Azuma* and Alicia A. McDonough. USC School of Medicine, Dept. of Physiology and Biophysics, L.A., CA 90033.

We have previously found that thyroid hormone (T3) increases Na,K-ATPase (NKA) α1 and β1 subunit mRNA levels, and is necessary for the euthyroid (eu) levels of expression of β1 mRNA, and α2 mRNA and protein levels in skeletal muscle. We had not determined the steady state hyperthyroid (hyper) levels of any of the subunit isoforms, nor did we detect α1, β1 nor β2 protein levels. The aims of the present study was to (1) detect α1, β1, and β2 protein in skeletal muscle and (2) demonstrate their regulation by T3. Specifically, we established the NKA subunit mRNA and protein levels in skeletal muscle at three steady states: hypothyroid (hypo), eu, and hyper.

Hypo rats treated with a 2 wk low iodine diet and 0.02% methylimidazole-2-thiol in the water. Hyper rats were hypos injected daily with 1 ug T3/g body wt for up to 16 days. mRNA levels were analyzed by dot blot hybridization and protein abundance by immunoblot with isoform specific probes.

We found that α1 and β1 mRNA and protein levels were not regulated by T3. α2 was highly regulated: mRNA reached a new steady state level of 5 fold over hypo by 8 days of T3 treatment and protein abundance increased by 3 fold. β2 mRNA and protein was detected and found to be similarly regulated by T3: mRNA increased nearly 4 fold over hypo levels and protein abundance increased over 2 fold. The hypo levels of α2 and β2 protein were decreased to 0.6 of the eu state.

In conclusion, we have detected β2 mRNA and protein in skeletal muscle. We have shown that both α2 and β2, not α1 nor β1 are highly regulated in skeletal muscle by T3, and the eu levels of expression are dependent on the hormone, suggesting that the T3-responsive enzyme in rat skeletal muscle is an α2:β2 dimer. (Supported by DK 34316, HL39295, HL08074 to KKA)

11.9

DISTRIBUTION OF NA,K-ATPASE α1, α2 AND ß1 PROTEIN LEVELS BETWEEN CARDIAC VENTRICLE AND SEPTUM. Charles B. Hensley*, Farid Nakhoul and Alicia A. McDonough. U.S.C. School of Medicine, Dept. of Physiology and Biophysics, L.A., Ca 90033.

Regional differences in Na,K-ATPase isoform distribution are likely to play an important role in the physiology of the myocardium. The effects of cardiac glycosides are especially prominent in the cardiac conduction system. Since Na,K-ATPase α2 and α3 isoforms are much more sensitive to cardiac glycosides, it is reasonable to assume that the conduction system may have a higher level of expression of these isoforms. It has recently been shown by *in situ* hybridization analysis that regions of the conduction system express higher levels of Na,K-ATPase α2 and α3 mRNA compared to ventricle (Zahler et al., 1992). Since the level of mRNA do not necessarily correlate with the level of protein, we aimed to determine the level of Na,K-ATPase α1, α2 and ß1 protein expression in both cardiac ventricle and septum.

Cardiac ventricle and septum were dissected from hearts of male Sprague Dawley rats (250g) for analysis of Na,K-ATPase protein subunit levels. Relative abundance of Na,K-ATPase subunits in ventricle vs septum was determined by immunoblot analysis of 50 ug protein/sample homogenates.

We found no major differences in the Na,K-ATPase isoform pool sizes between the cardiac ventricle and septum. We conclude that the protein levels measured by immunoblot do not correspond to the mRNA levels suggested by in situ hybridization and that the differences seen at the level of mRNA may be due to high turnover rates of α2 in cells of the conduction system. (Supported by AHA-GLAA Senior Investigatorship to CBH)

11.10

ONTOGENY OF NaK-ATPase α_1 ISOFORM mRNA IN MOUSE LUNG EPITHELIUM DURING THE PERINATAL PERIOD. R.G. Crump*, C.H. Joiner, G.R. Askew*, and S.F. Wert*. Divisions of Neonatology and Pulmonary Biology, Children's Hosp. Med. Center; Dept. of Molecular Genetics, Biochemistry, & Microbiology, Univ. of Cincinnati College of Med., Cincinnati, Ohio 45229

Fetal lung fluid is removed at birth by mechanisms thought to involve epithelial ion transport driven by NaK-ATPase. We postulated that NaK-ATPase α_1 isoform mRNA would localize predominantly to airway epithelium and would increase perinatally. Slot blot assays of whole lung mRNA revealed constant levels of α1 mRNA during the perinatal period. *In situ* hybridization with radiolabeled RNA probe specific for the α1 isoform was used to determine tissue distribution and relative levels of α_1 mRNA from fetal day (FD) 14 to neonatal day (ND) 1 (<24 hrs old). Alpha$_1$ iso-form mRNA was higher in proximal airway epithelium compared to distal parenchymal structures. A substantial increase in signal in airway epithelium was noted between FD 18.5 and ND 1. Con comitantly, α_1 isoform mRNA also increased in pulmonary veins, which contain cardiac myocytes in mouse. The increase in airway epithelial NaK-ATPase mRNA is consistent with previous studies showing increased Na transport perinatally, suggesting that the airway epithelium plays an important role in fluid resorption at birth.

11.11

MOLECULAR CHARACTERIZATION OF THE Cl^- PUMP. George A. Gerencser. Department of Physiology, College of Medicine, University of Florida, Gainesville, FL 32610.

The serosa negative transepithelial potential difference across *Aplysia* gut is generated by a Na^+ and HCO_3^--independent active electrogenic Cl^- absorptive mechanism. Lending credence to the idea that the Cl^- transport process is a Cl^- pump (i.e., a primary active transport mechanism) were the observations of; 1) the existence of Cl^--stimulated ATPase activity and ATP-dependent Cl^- accumulation in *Aplysia* foregut absorptive cell basolateral membrane vesicles, both of which were inhibitable by vanadate, and 2) reconstitution of both of these activities into preformed liposomes, and again, both of which were inhibitable by vanadate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the Cl^- pump-containing proteoliposomes yielded two protein bands, one of which was a major band at 116 K daltons and the other being a minor band at 97.5 K daltons. Also, these purified proteins of the Cl^- pump have been subjected to phosphorylation within the proteoliposome and the reaction sequence and kinetics of the reaction sequence of the enzyme have been determined: Mg^{2+} causing phosphorylation, Cl^- causing dephosphorylation, and all in a time frame consistent with an acyl phosphate linkage. Hydroxylamine and high pH destabilize this phosphorylation. Orthovanadate (10^{-7} M) almost completely inhibits the Mg^{2+}-driven phosphorylation reaction. This demonstration of reconstitution of the Cl^- pump and its ascertained molecular weight and phosphorylated reaction sequence provides the first direct evidence for the existence of a new "P" type ATPase: The Cl^- pump. Supported by the Eppley Foundation for Research, Inc.

11.12

PROTONMOTIVE FORCE ACROSS THE APICAL MEMBRANE OF MALPIGHIAN TUBULES OF *FORMICA* DRIVES ACTIVE K EXTRUSION IN THE LUMEN. P. S. Steels*, S-L Zhang*, A. Leyssens* and E. Van Kerkhove*, (SPON: W. Van Driessche). Dept. MBW, Limburgs Universitair Centrum, 3590 Diepenbeek, Belgium.

Malpighian tubules of *Formica* actively secrete K. It was shown that a V-type H-ATPase is involved in the active transport step at the apical membrane (Weltens et al., Cell Physiol. Biochem., 1992;2:101-116). This electrogenic H-pump is supposed to be the prime mover in active K transport: the proton concentration gradient built up across the apical membrane drives a K/H exchanger, realizing a net active and apparent electrogenic K flux into the lumen. In the present study the total electromotive force across the apical membrane,(the apical membrane potential,V_{ap}, minus the Nernst potential,E_{ap}), for both H and K was determined in different bath K concentrations (5, 51 and 113 mM), known to be positively correlated with secretion rate (J_V). The following results for J_V, V_{ap}-E_H,and V_{ap}-E_K were obtained in the three K concentrations, respectively: 72, 103 and 149 pL/min; -122,-103 and -87 mV; -99, -57 and -44 mV. From the plot of Jv versus the electromotive force the reversal potential of the pump was estimated to be - 153 mV, which corresponds to a $\Delta G^{ap/H}$ of 3.5 Kcal/mol. If the molar free energy of hydrolysis of ATP is supposed to be 12 Kcal/mol, a H/ATP stoichiometry of at the least 3 can be calculated. It is concluded that the electromotive force of H across the apical membrane can drive the K extrusion in the lumen and the H pump does not operate at equilibrium in normal conditions.

11.13

MUTATIONS IN THE *b* AND δ SUBUNITS OF THE *E. COLI*. PROTON TRANSLOCATING F_1F_0 ATP SYNTHASE. K. A. McCormick, A. E. Stack, and B. D. Cain (SPON: M. S. Kilberg). University of Florida, Gainesville, FL 32610

The *b* and δ subunits of ATP synthase participate in the coupling of ion transport through the membrane intrinsic F_0 portion of the enzyme to catalysis occurring in F_1. Site-directed mutagenesis was used to define amino acids of the *b* and δ subunits which are important for enzyme function. Alanine-79 of the *b* subunit is sensitive to mutation (McCormick and Cain, *J. Bacteriol* 173: 7240-7248). A variety of polar and hydrophobic amino acids were introduced at position 79. *In vivo* enzyme activity was significantly reduced by the replacement of Ala-79 with Pro, Glu, Lys, and Gln. Mutant strains were assayed for ATP hydrolysis driven proton pumping and membrane associated ATP hydrolysis. The results suggest that loss of enzyme function is correlated with a defect in the association of F_1 to F_0. A mutagenic analysis of the δ subunit focused upon Asp-161 and Ser-163. Growth yields of several mutant strains were reduced relative to wild type strains, indicating a marked impairment of enzyme function. The disruption of interactions between F_1 and F_0 subunits observed for the *b* and δ subunit mutations suggests a reduction in the capacity of the enzyme complex to couple ATP synthesis to proton translocation.

11.14

MUTAGENIC ANALYSIS OF THE CARBOXYL-TERMINAL REGION OF SUBUNIT *a* IN *E. coli* F_1F_0 ATP SYNTHASE. P. Hartzog and B.D. Cain (SPON: M.S. Kilberg). Univ. of Florida, Gainesville, FL 32610

Subunit *a* of the *E. coli* proton translocating F_1F_0 ATP synthase contains a region of striking primary sequence homology with the mitochondrial and chloroplast enzymes. The area of homology is near the carboxyl-terminus and is thought to be important in ion conductance. Cassette site-directed mutagenesis has been used to study the functional roles of conserved amino acids in proton transport. Amino acid substitutions at position *gln*252 exhibited effects on F_1F_0 ATP synthase activity consistent with this position having a critical structural role in F_0. The structurally conservative *gln*252→*glu* mutation resulted in a minimal effect on ATP synthase activity while the *gln*252→*leu* and *gln*252→*val* mutations exhibited significantly reduced enzyme activity. The *gln*252→*lys* mutation resulted in an apparent loss of F_0 proton translocation activity as shown by studies of both ATP-driven proton pumping and proton permeability of stripped membranes. Amino acid substitutions at the three positions *phe*256, *leu*259 and *tyr*263 also resulted in the reduction of F_1F_0 proton translocation. These positions showed more flexibility to mutation unless substituted with a charged amino acid. The *phe*256→*arg* and *leu*259→*arg* mutations resulted in the apparent loss of proton translocation while the *phe*256→*asp* and *tyr*263→*arg* mutations only reduced proton transport. The data are most consistent with the hypothesis that the region between *gln*252 and *tyr*263 of the *a* subunit has an important structural role in forming the ion translocating mechanism of F_0.

11.15

EXPRESSION CLONING OF HUMAN AND RAT RENAL CORTEX Na/P_i-COTRANSPORT. Simona Magagnin*, Andreas Werner*, Victor Sorribas* Jürg Biber* Heini Murer. Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland.

We have recently cloned a protein which is most likely involved in rabbit renal cortex Na/P_i-cotransport (NaPi-1; A. Werner et al. PNAS, 88, 9608; 1992). Northern blot hybridization experiments using a corresponding cDNA probe provided no basis for a 'homology-based' cloning of the 'related' human and rat transport systems. Therefore, we have reused an expression cloning strategy, using the XENOPUS laevis (XL)-system.

Injection of size fractionated mRNA (2-3 kB; either from human or rat kidney cortex) led to a several-fold stimulation of Na-dependent P_i-uptake in XL-oocytes. cDNA-library constructions followed by sib-selction procedures resulted in the isolation of a human and rat cDNA-clone, respectively, which after *in-vitro* transcription (cRNA) led to specific stimulation of Na-dependent P_i-transport in XL-oocytes; injection of 1 ng of cRNA resulted after 4 days of incubation in a 30 to 50-fold stimulation of Na-dependent P_i-transport. Transport stimulation was specific for P_i and not observed for transport of either sulphate, L-arginine, L-leucine or D-glucose. Kinetic analysis of expressed transport activity (for human and rat cRNA, respectively) resulted in an apparent K_m for P_i of around 100-150 μM and for Na of ≈ 50 mM (Hill-coefficients above 2). The cDNA inserts (and corresponding cRNAs) are about 2.7 kB in length and encode for proteins of ≈ 60-70 kDa (*in-vitro* translation).

On the basis of above results we assume to have structurally identified two 'novel' proteins which are most likely involved in renal (proximal tubular brush border membrane) Na/P_i-cotransport in humans and rats, respectively.

11.16

LOCALIZATION OF A Na/P_i-COTRANSPORT SYSTEM IN THE RABBIT NEPHRON BY IMMUNOHISTOCHEMISTRY AND RT-PCR. Jürg Biber*, Maria Custer*, Brigitte Kaissling*, Arlyn Garcia-Perez, Andreas Werner* and Heini Murer. Institute of Physiology, University of Zürich/Switzerland and NHLBI, NIH, Bethesda/MD/USA

Localization of a recently cloned Na-dependent transport system for phosphate (NaPi-1; A.Werner et al., PNAS 88:9608;1992) has been carried out in the rabbit kidney at the level of the expressed protein (immunohistochemistry) and the corresponding mRNA (RT-PCR).

For immunohistochemistry a polyclonal antibody raised against a C-terminal synthetic peptide of NaPi-1 has been used. Specifity of this antibody is documented by Western blots using isolated proximal brush border membranes and immunoprecipitation of *in-vitro* translated NaPi-1. Specific (peptide protectable) immunofluorescence was observed in the apical membrane of proximal tubular cells; no specific staining was observed in the other segments including glomeruli.

In microdissected nephron segments NaPi-1 specific mRNA was amplified by PCR after reverse transcription (RT-PCR). As a control, mRNA coding for ß-actin was amplified in parallel. NaPi-1 specific (RT-dependent) PCR products were obtained from proximal, but not from other tubular segments.

We conclude that NaPi-1, a cloned Na/P_i-cotransporter from rabbit kidney cortex represents a Na/P_i-cotransport system of the apical membrane of the proximal tubular cell.

11.17

EXPRESSION CLONING OF RAT RENAL BRUSH BORDER NA/SO_4 COTRANSPORT. Daniel Markovich*, Andi Werner* and Heini Murer. Institute of Physiology, University of Zürich, CH-8057 Zürich, Switzerland.

Brush border membrane Na/SO_4 cotransport is an essential step in proximal tubular sulphate reabsorption. For a structural identification of this transport system, we have used a functional cloning strategy based on expression cloning in *Xenopus laevis* oocytes. Injection of rat kidney cortex mRNA into stage VI oocytes led to a dose and time-dependent response, with the expression of a Na-dependent uptake of sulphate (Na/SO_4 cotransport). Maximal stimulation was reached with 25ng of injected mRNA at day 4 post injection, showing a 3-fold stimulation over background activity (water injected oocytes). Size fractionation of this mRNA through a sucrose density gradient gave rise to an average mRNA size of 1.8-2.4kb, which displayed a 6-fold stimulation of Na/SO_4 cotransport over background. A cDNA library was constructed using the size fractionated mRNA and was screened (in-vitro cRNA transcripts injected into oocytes) using standard sib selection procedures. A single positive clone was isolated encoding a protein which specifically stimulates Na-dependent sulphate uptake. We assume to have cloned a rat renal Na/SO_4 cotransporter.

11.18

ROLE OF BLOOD PRESSURE IN REGULATING Na^+/H^+ EXCHANGE OF VASCULAR SMOOTH MUSCLE - EFFECT OF STRAIN DIFFERENCES. Cheryl R. Scheid, Deborah R. Ellstrom* and Thomas W. Honeyman. Univ. of Massachusetts Med. Sch., Worcester, MA 01655.

Na^+/H^+ exchange differs kinetically in segments of mesenteric arteries from spontaneously hypertensive rats (both transport rate and sensitivity to $[H^+]_i$ are greater in SHR than in normotensive WKY controls). These kinetic changes are not seen in prehypertensive SHR or in SHR rendered normotensive by captopril therapy; thus blood pressure appears to be important for regulating Na^+/H^+ exchange in SHR. To assess the effects of elevated pressure on the WKY transporter, WKY rats were rendered hypertensive by treatment with DOCA/salt; and Na^+/H^+ exchange was examined in mesenteric arteries. DOCA/salt treatment (injection of 30 mg/kg deoxycorticosterone acetate twice per week for 4 weeks, 1% NaCl in the drinking water) produced a significant increase in mean arterial pressure (181 ± 29 *vs* 133 ± 15 in DOCA/salt *vs* salt alone, n= 8 and 3, respectively). This treatment had no effect on either the maximal transport rate or on the sensitivity to $[H^+]_i$ (measured as the Hill coefficient). V_{max} averaged 0.103 $\pm$ 0.011 *vs* 0.093 $\pm$ 0.006 ($p > 0.05$) and Hill averaged 4.4 (R value for fitted line = 0.95, data from 11 strips) *vs* 4.4 (R = 0.95, data from 4 strips) in vessels from hypertensive (DOCA/salt) *vs* normotensive (salt) animals. Thus elevated pressure has differing effects in different rat strains, suggesting strain differences in the transport protein or its regulation. *Supported by NIH HL41188.*

11.19

MULTIPLE CARBOHYDRATE MOIETIES ON THE Na+/H+ EXCHANGER Larry Fliegel* and Robert S Haworth* (SPON: O. Froehlich) University of Alberta, Edmonton, AB, T6G 2S2, Canada.

Affinity purified antibodies against the carboxyl-terminal region of the Na+/H+ exchanger were used to analyze the structure and character of the carbohydrate moiety of the amiloride sensitive Na+/H+ exchanger. The amiloride sensitive Na+/H+ exchanger (NHE-1) in human placental brush border membranes has an approximate molecular weight of 105-kDa. Incubation of both intact brush border membrane vesicles and detergent-solubilised membranes with glycopeptidase F removed the carbohydrates and increased the apparent mobility of the exchanger to 98-kDa. Digestion with Endo-F caused a similar change, while Endo-H had no effect suggesting that the placental Na+/H+ exchanger is a glycoprotein of the biantennary complex type. Removal of the carbohydrate moiety with glycopeptidase F had no effect on the ability of the protein to promote the exchange of Na+ for H+ and had no detectable effect on the sensitivity of the exchanger to trypsin. Limited digestion with glycopeptidase F indicated the presence of 2 intermediate forms between the fully glycosylated and the deglycosylated protein. This suggests the presence of at least two, and possibly three, N-linked carbohydrate moieties. The results suggest that the amiloride sensitive Na+/H+ exchanger is a glycoprotein with at least two carbohydrate groups of the biantennary complex type. Supported by MRC of Canada

11.20

Rat proximal tubule Na^+/Ca^{2+} exchanger gene expresion in toxicity. J.H. Dominguez*, M. Oulali*, K.Camp*, C.C. Hale*, S.B. Kleiboeker*, and H. Feister* (SPON: T.D.McKinney). VAMC and IUMC, Indianapolis, IN, 46202.

Gentamicin (G) toxicity (Tox) causes cell Ca^{2+} accumulation in proximal tubules (PT), an effect that may result from reduction of Na^+/Ca^{2+} exchanger (NaCaX) activity, an important Ca^{2+} efflux system (AJP 91). PT NaCaX gene expression was evaluated in control (C) and G-treated rats (GRx), 40 mg/kg twice d for 10 d (injury = I), and after healing for 15 d (H). *Serum creatinine* in I was higher in GRx than C (1.38 ± 0.35 vs 0.42 ± 0.02, mg/dl, n=6, $p<0.02$), and improved in H, 0.55 ± 0.03, $p<0.05$). In GRx PT NaCaX mRNA, normalized to 28S rRNA, was decreased in I to only 63 $\pm$ 12 % of C ($p=0.02$) and improved in H to 72 ± 15 % ($p=0.1$, n=4). Specific NaCaX protein level was also reduced in GRx to 67 ± 10 % of C and improved to 82 ± 10 % in H. NaCaX activity in GRx PT homogenates was only 40 ± 16 % of C ($p<0.05$). G Tox on PT α_1-subunit of Na^+/K^+ ATPase (α_1) and GLUT 1 genes was also tested to verify specificity. Remarkably, α_1 mRNA and protein levels were not affected in GRx, and GLUT 1 mRNA actually increased by 43 ± 9 % ($p<0.05$). To test if inhibition of NaCaX gene expression by G Tox preceded renal failure, C & GRx were studied for 1,3, and 8 days. Creatinine was comparable in both groups for each day; and after 8 days of G Tox: 0.45 ± 0.03 & 0.50 ± 0.06 ($p > 0.05$). However, NaCaX protein level was reduced to 68 % of C, and functional activity by 20 ± 3 % ($p<0.01$). We conclude: (i) G toxicity suppresses NaCaX gene expression with some specificity. (ii) The toxicity of G on NaCaX precedes renal failure. (iii) GLUT 1, a potential stress gene, is activated in PT by G injury.

11.21

IDENTIFICATION OF REGULATORY REGIONS OF THE CARDIAC SARCOLEMMAL Na^+-Ca^{2+} EXCHANGER. D.A. Nicoll*, S. Matsuoka*, R.F. Reilly*, D.W. Hilgemann*, and K.D. Philipson. UCLA, Los Angeles, CA 90024; Univ. Texas, Southwestern, Dallas, TX 75235; Yale, New Haven CT 06510

We have analyzed the regulatory properties of the wild-type cardiac exchanger expressed in *Xenopus* oocytes using the giant excised patch technique. The expressed exchanger displays the same properties observed in the native sarcolemmal exchanger: Activation by chymotrypsin, Na^+-dependent inactivation, secondary regulation by intracellular Ca^{2+}, and inhibition by a specific exchanger inhibitory peptide (XIP). These properties were localized to the large hydrophilic domain of the exchanger by deletion mutagenesis and construction of a chimeric exchanger. Analysis of the mutants demonstrated that residues 240-679 are not essential for ion transport; that residues 562-679 are involved in regulation by Ca^{2+}; and inhibition by XIP; that Na^+-dependent inactivation is separable from secondary Ca^{2+} regulation and localized to residues 240-561; and that the two hydrophobic domains of the exchanger are involved in ion binding and transport. A chimera made from renal and cardiac exchangers helps to further define the residues involved in Ca^{2+}-regulation and XIP interaction.

11.22

EXPRESSION OF HUMAN ANION TRANSPORTER AE1 (BAND 3) IN DICTYOSTELIUM DISCOIDIUM. R. B. Gunn, P. Smith and K. Liu. Emory Univ. Atlanta, GA 30322-3110.

The human band 3 cDNA was inserted into the Dictyostelium discoideum(Dd) expression vector pBS18 with codons(#1-5) mutated to those preferred by Dd. Only two transfections have grown G418 resistant cultures with elevated 36-Cl influxes when suspended in HL5 media (Cl=20mM) at pH=7.4 at 20C. These two polyclonal cultures were grown, transfected and selected in 20uM DNDS, dinitrostilbenedisulfonate, a reversible inhibitor. Control and transfected cells without the band 3 cDNA in the vector have initial influx rates of 0.5-2 pmoles/ug protein min. The expressing cells had fluxes of 5-13 pmoles/ug protein min. Neither this elevated flux nor the control flux was significantly reduced by DNDS added to the influx media. But in a single experiment when the expressing cells were grown for 48 hours without DNDS the flux did not increase further but was DNDS inhibitable to the control level. The flux Q10 was 2; the phloretin inhibition equivocal. The cells were positive by Southern and northern analysis but negative by western analysis, but the incremental influx could be due to as few as 100 copies of band 3 per cell. After 3 months all cultures became negative by flux and northern analysis. HL28674.

11.23

REGULATION OF ENDOTHELIAL CELL Na-K-Cl COTRANSPORT BY EXTRACELLULAR TONICITY: EFFECTS OF PHOSPHATASE AND KINASE INHIBITORS. M. O'Donnell. University of California, Davis, CA 95616

Vascular endothelial cells possess a highly active Na-K-Cl cotransport system that is stimulated by extracellular hypertonicity and appears to be important for cell volume regulation. Vasopressin and other agents that stimulate endothelial cotransport appear to act via a Ca-dependent pathway. However, previous studies indicate that stimulation of cotransport by hypertonicity is Ca-independent and does not involve changes in levels of cyclic AMP, cyclic GMP or protein kinase C activity. The present study was conducted to further investigate the regulation of endothelial Na-K-Cl cotransport by hypertonicity. To test the possibility that hypertonicity-induced stimulation of cotransport involves a phosphorylation event, the effect of the phosphatase inhibitor okadaic acid was evaluated. Na-K-Cl cotransport activity was assessed as bumetanide-sensitive ^{86}Rb uptake in both bovine aortic and pulmonary artery endothelial monolayers. Okadaic acid (1 μM) was found to stimulate cotransport activity of the endothelial cells both under basal (isotonic) and hypertonic (400 mOsm) conditions. The myosin light chain kinase inhibitor ML-7 was also evaluated for its effect on cotransport in the present study. Treatment of the endothelial cells with ML-7 (0.1 μM) was found to inhibit hypertonicity-induced stimulation of endothelial Na-K-Cl cotransport but to have no effect on basal or vasopressin-stimulated cotransport. ML-7 was also found to inhibit the volume regulatory response of endothelial cells exposed to hypertonicity. These findings suggest that stimulation of endothelial Na-K-Cl cotransport by hypertonicity may be mediated by a mechansim involving myosin light chain kinase. Supported by NIH 45674 and Am. Heart Assoc. 901187.

11.24

PRIMARY STRUCTURE AND CHARACTERIZATION OF THE THIAZIDE-SENSITIVE Na^+:Cl^- COTRANSPORTER (TSC) OF THE WINTER FLOUNDER URINARY BLADDER. Gerardo Gamba*, Akihiko Miyanoshita*, Michael Lombardi* and Steven C. Hebert. Brigham and Women's Hospital. Boston, MA 02115

The winter flounder (*Pseudopleuronectes americanus*) urinary bladder transports Na^+ via an electroneutral, thiazide-sensitive Na^+:Cl^- cotransporter. We used a functional expression strategy in *Xenopus laevis* oocytes to screen a flounder bladder cDNA library until a single clone (3.7 kb) that expressed cotransporter activity was identified (TSC_{fl}). Injection of cRNA from the TSC_{fl} clone into oocytes gave rise to a Cl^--dependent $^{22}Na^+$ uptake with the following characteristics: 1) Na^+ and Cl^- interdependence (stoichiometry of 1:1); 2) Km values for Na^+ and Cl^- of 25 ± 0.4 and 13 ± 0.2 mM; 3) sensitivity to thiazide diuretics (K_i for metolazone = 3 μM); and 4) insensitivity to bumetanide, amiloride, acetazolamide and EIPA. The TSC_{fl} cDNA sequence revealed an open reading frame of 3,069 bp that predicts a protein of 1023 amino acids residues with calculated Mr of 112 kDa and 12 putative membrane spanning segments. No significant similarities were found in searches of genetic databases. SDS-PAGE of *in vitro* translation products showed a single band of 112 kDa in the absence and three bands in the presence of membrane: 121 and 97 kDa glycosylated (112 and 85 kDa after treatment with endoH) and 25 kDa non-glycosylated. Northern analysis at high stringency of several flounder tissues using a full-length probe showed a single bands of 3.7 kb in the urinary bladder and 3.0 kb in gonad, intestine, skeletal muscle, eye, brain and kidney. Using shorter probes from the TSC_{fl} subclones we observed that ~ 700 bp at the 5' end of TSC_{fl} are absent in the non bladder gene products.

In conclusion, we have cloned and sequence a cDNA encoding the thiazide-sensitive $1Na^+$:$1Cl^-$ cotransporter from the winter flounder bladder. *In vitro* translation studies suggest that, in the presence of membrane, TSC_{fl} protein is cleaved into two polypeptides: 97 and 25 kDa. The 5' alternative spliced form expressed in non-bladder tissues may represent the 97 kDa glycosylated product of TSC_{fl}.

11.25

TISSUE DISTRIBUTION OF THE Na-K-Cl COTRANSPORTER IN THE SPINY DOGFISH, *Squalus acanthias*. John A. Payne, Daniel Biemesderfer*, Christian Lytle*, Jian-Chao Xu*, John Donald*, and Bliss Forbush III*. Mt. Desert Is. Biol. Lab. Salsbury Cove, ME 04672, Depts. of Cellular and Molecular Physiology and Internal Medicine, Yale University School of Medicine, New Haven, CT 06510, and Dept. of Zoology, University of Florida, Gainesville, FL 32611.

The distribution of the Na-K-Cl cotransporter was examined in various tissues of the spiny dogfish, using both monoclonal antibodies (mAbs) and cDNA probes specific for this transport protein. Four mAbs have been developed which identify the 195 kDa Na-K-Cl cotransport protein from shark rectal gland (Forbush and Haas, Biophys. J., 53:222a, 1988). Treatment with N-glycanase reduces the molecular weight of this protein to ~137 kDa, indicating that a significant portion of the apparent mass is carbohydrate. In membranes from shark brain and shark eye, a 160 kDa protein was detected by the mAbs. On deglycosylation with N-glycanase, the protein was indistinguishable in size from the deglycosylated rectal gland protein. In microsomes from other tissues (e.g., gill, intestine, kidney, stomach), a ~250 kDa protein was the only protein detected, and this protein was resistant to N-glycanase. Immunohistochemical studies demonstrated specific immunostaining in salt transporting epithelia (e.g., rectal gland, kidney, gill). Northern blot analysis, using cDNA probes encoding the rectal gland cotransporter, revealed a 7.3 kb band in rectal gland and a 7.0 kb band in brain. These results suggest that there are different forms of the Na-K-Cl cotransporter in different tissues which vary in glycosylation state and possibly amino acid sequence. (Supported by NIH grant DK-17433)

11.26

Na^+- AND K^+-DEPENDENT BASOLATERAL Cl^- TRANSPORT IN SURFACE EPITHELIUM OF NECTURUS ANTRUM. **D.I. Soybel and M.B.E. Davis*, Yale Univ. Sch. of Med. and V.A.M.C., West Haven, CT 06516.**

Preliminary studies suggest that basolateral Cl^- entry into the gastric surface cell is reduced when Na^+ is removed from the nutrient solution and increased when serosal [K^+] levels are increased. The goal of this study was to evaluate whether K^+-dependent Cl^- entry depends on the presence of serosal Na^+ and whether such entry might be reduced by bumetanide, a blocker of Na/K/Cl co-transport. Antral mucosae of Necturus were mounted in Ussing chambers, perfused serosally by amphibian Ringer (Na 108 mM, Cl 94 mM). To prevent interference by luminal Cl^-, all Cl^- in the luminal perfusate was replaced by gluconate. Intracellular microelectrodes were used to measure cell membrane potentials (V_c) and intracellular Cl^- activity (a^i_{Cl}). V_c and a^i_{Cl} were measured during the change from low [K^+](4 mM) to high [K^+](60 mM): 1) under control conditions (n=15); 2) when all Na^+ was replaced in the serosal solution by NMDG$^+$ (n=7); 3) when control levels of Na^+ and 10^{-4}M bumetanide were present in the serosal solution (n=8). Results (means±SE, *p<0.05 compared to low K):

	Control		0 Na^+		Bumetanide	
	low K^+	high K^+	low K^+	high K^+	low K^+	high K^+
V_c(mV)	-44±3	-19±2*	-46±5	-20±4*	-43±3	-14±2*
a^i_{Cl}(mM)	13±2	22±3*	9±1	12±2	11±1	15±2*

Under control conditions, raising serosal [K^+] increased a^i_{Cl} markedly. These increases were not observed in the absence of serosal Na^+ and were significantly ($p<0.05$) reduced by bumetanide. Na/K/Cl co-transport may regulate a^i_{Cl} in this tissue.

11.27

CHANGES IN CATION TRANSPORT FOLLOWING ELEVATION OF CYTOPLASMIC CALCIUM IN CULTURED HUMAN NON-PIGMENTED CILIARY EPITHELIAL CELLS. Nicholas A. Delamere, Takezo Mito and *Miguel Coca-Prados. Dept of Ophthalmol and Vis Sci, KY Lions Eye Res Inst, Univ of Louisville Sch of Med, Louisville, KY 40292 and *Dept of Ophthalmology, Yale University School of Medicine, New Haven, CT 06510

Cytoplasmic Ca^{2+} mobilization may mediate hormone-induced changes in aqueous humor secretion by ciliary epithelium. Because secretion is linked to ion transport, we performed ^{86}Rb uptake studies to test whether Ca^{2+} changes Na,K-ATPase and Na/K/2Cl co-transporter activity. In a cell line (ODM2) derived from human non-pigmented ciliary epithelium, elevating intracellular Ca^{2+} by A23187 or thapsigargin increased both ouabain-sensitive K (^{86}Rb) uptake (Na,K-ATPase-mediated) and ouabain-insensitive, bumetanide-sensitive K (^{86}Rb) uptake (Na/K/2Cl co-transporter-mediated). The increases could be prevented by the intracellular Ca^{2+} buffer BAPTA-AM. A23187 also increased the ^{86}Rb efflux rate in a manner that could be blocked by quinidine (0.1 mM). We propose that activation of quinidine-sensitive K channels is involved in the Ca^{2+}-induced stimulation of Na,K-ATPase since quinidine also blocked the A23187-induced increase of the ouabain-sensitive portion of K (^{86}Rb) uptake. However, Ca^{2+}-induced stimulation of Na/K/2Cl co-transport may not require K channel activation. (Supported by NIH grant EY06915)

11.28

CELLULAR MECHANISM OF ETHANOL ACTION ON RENAL TUBULAR TRANSPORT. Tong Wang* and Yun Lai Chan. Univ. of Illinois Col. of Medicine, Chicago, IL 60612.

Ethanol induced diuresis is believed to be a result of inhibiting vasopressin release. Recent evidence, however, has demonstrated that a moderate dose of ethanol administered intraperitoneally to the rat can stimulate vasopressin release. Thus, another mechanism in addition to vasopressin release may be responsible for the diuretic effect of ethanol. This study was designed to examine the direct effect of ethanol on renal tubular transport by using microperfusion techniques. Proximal convoluted tubule (PCT) and peritubular capillaries (CP) were perfused with Ringer solution in situ in the rat kidney. Bicarbonate was determined by microcalorimetry and ^3H-inulin was used as a volume marker. The rate of bicarbonate absorption ($JHCO_3$) was 140.8 ± 4.5 pEq/min x mm and fluid absorption (Jv) was 2.51 ± 0.15 nl/min x mm. The addition of ethanol (25 mM) to the PCT perfusate caused reductions in $JHCO_3$ and Jv. A higher dose (50 mM) of ethanol, in contrast, caused increases in $JHCO_3$ and Jv. Similar results were obtained when ethanol was added to the CP perfusate in both innervated and denervated kidney.

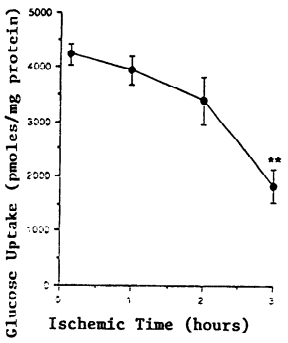
Our results suggest that ethanol has an effect on Na^+/H^+ exchange. The cellular mechanism of ethanol action may be correlated to the changing of intracellular calcium.

11.29

MEMBRANE TRANSPORT IN ASSESSMENT OF INTESTINAL ISCHEMIA

A.Sisley*, E.Ng*, B.Gewertz, J.Harig*
University of Chicago, Chicago, IL 60637

Intestinal ischemia is traditionally evaluated with mucosal histology. We hypothesized that degradation of membrane transport functions, assessed with purified intestinal brush border membrane vesicles (BBMV) would precede detectable histologic changes. Small bowel, harvested from 10 male mongrel canines, was placed in warm saline and sampled at 0, 1, 2 and 3 hr. of ischemia. Mucosal biopsies were graded blindly on a scale of 1 (normal) to 5 (necrosis of the entire villus). Mucosal BBMV were assayed for Na^+-Glucose cotransport by incubation with ^{14}C-Glucose. Histologic grades were unchanged, (1 at time 0, 1.5 at 3 hr.) while Na^+-Glucose cotransport showed a significant decrease at 3 hr. of ischemia ($p<.05$)**. Intestinal BBMV transport provides a more sensitive means of quantitating ischemia than mucosal histology. (Supported by American Heart Association grant entitled Mechanism of Reperfusion Injury in Human Intestines.)



SYSTEMIC AND INTEGRATIVE PHYSIOLOGY

12.1

PRESSOR RESPONSES TO ANGIOTENSIN II AND NOREPINEPHRINE IN SPONTANEOUSLY HYPERTENSIVE RATS FED GAMMA-LINOLENIC ACID RICH OILS. Marguerite M. Engler, University of California, San Francisco, CA 94143-0610.

Recent studies by this laboratory have shown that gamma-linolenic acid (GLA) rich oils produce an antihypertensive effect in spontaneously hypertensive rats (SHR). A potential mechanism for such an effect may be an alteration in pressor responsiveness to vasoconstrictor hormones. To examine this possibility, male SHR (6-7 wks old) (n=41) were fed for 7 wks a purified diet with 11% wt of either sesame oil (control), evening primrose oil (8% GLA), blackcurrant oil (16% GLA) borage oil (19% GLA), or fungal oil (25% GLA). The animals were anesthetized with halothane and the femoral artery and vein cannulated. Intraarterial pressor responses to graded intravenous infusion of angiotensin II (AII) (1.25-80 ng/kg) and norepinephrine (NE) (125-2000 ng/kg) were determined. A dose-dependent increase in blood pressure was observed for AII and NE in each dietary group. However, the pressor responses to AII and NE were not significantly different between dietary groups. These findings suggest that the blood pressure lowering effect of GLA rich oils in SHR cannot be explained by altered sensitivity to vasoconstrictor hormones. Therefore, other mechanisms should be investigated.

12.2

AN EXPLORATORY STUDY ON THE EFFECT OF ANAEROBIC EXERCISE UPON THE TOTAL SERUM CHOLESTEROL LEVEL OF COLLEGE-AGE FEMALES. J. Richardson, L. Tentinger*, M. Hoadley*, M. Stevenson*, P. Vitale*. University of South Dakota, Vermillion, S.D. 57069

The purpose of this study was to investigate the effect of a six-week anaerobic conditioning program upon the total serum cholesterol level of college-age female participants. A single group pre-test/ post-test design was utilized in analyzing the existing data for this study.

The female subjects for this study consisted of the entire population of a 1992 college softball team (N=19). The mean age of the subjects was 19.47 years (s.d. = 1.07); the mean height was 66.21 inches (s.d. = 1.87); and the mean weight was 152.42 pounds (s.d. = 21.21).

The subjects participated in a six-week anaerobic softball conditioning program, whereby all workouts were conducted at the same time each day. The general guidelines for the conditioning program included a five-minute warm up and stretching period, followed by a 45-minute period of intermittent throwing, pivoting, and base running drills. Weight training was also a component of the conditioning program, as well as a five-minute cool down period. The subjects frequency of training was six days per week, with each training bout being approximately 90 minutes in duration.

Total serum cholesterol was evaluated after eight hours fast and rest for pretest and posttest utilizing the standard technique prescribed by the Mannheim Reflotron TM. All results were recorded in mg/dl.

The statistical data from the study was analyzed utilizing the Wilcoxon signed-ranks test for matched pairs. This nonparametric testing procedure involved comparing pretest and posttest total cholesterol level values for each subject.

The results of the study indicate that there was not a significant effect ($T_{cv} = 46$; $T = 72$) of a six-week anaerobic conditioning program upon the total serum cholesterol level of college-age female participants. Therefore, it appears that anaerobic exercise does not affect total serum cholesterol in college-age females.

12.3

RESUSCITATION OF ISCHEMIC MYOCARDIUM WITH ADENOSINE **Jaime Ponce*, Allen Garrett*, Eric Manahan*, and Race L. Kao.** East Tennessee State University, Johnson City, TN 37614.

Recovery of ischemic myocardium requires long duration. It is hypothesized that during reperfusion adenosine can be used to enhance the recovery of metabolic and contractile functions. A total of 48 male Sprague-Dawley rats (300-350g) were used for the double-blind study. Hearts were subjected to 45 minutes of normothermic global ischemia. During reperfusion, the treated group received perfusate containing adenosine (200 μM) for 5 minutes initially and at hourly intervals under retrograde perfusion. Hemodynamic performance and metabolic function were measured for each heart.

REPERFUSION	TREATMENT	AO	SBP	DBP	TAN
ONE HR.	ADENOSINE	16±3*	101±5*	45±2*	26±4*
	CONTROL	0±0	64±1	57±1	19±1
TWO HR.	ADENOSINE	18±3*	96±7*	52±1*	24±2*
	CONTROL	1±1	66±2	57±1	16±1
THREE HR.	ADENOSINE	19±4*	103±9*	50±3	25±3*
	CONTROL	0±0	65±1	60±1	10±1

AO=Aortic Output (ml/min), SBP=Systolic Blood Pressure(mmHg), DBP=Diastolic Blood Pressure (mmHg), TAN=Total Adenine Nucleotide (μM/g heart protein), *=p < .05 vs Control.

Adenosine significantly enhanced the recovery of ischemic myocardium during reperfusion. Adenosine can be used to benefit patients after acute myocardial infarction and those patients requiring circulatory assistance.

12.4

THE VASODILATOR PROFILE OF DOCOSAHEXAENOIC ACID: COMPARISON TO CYCLIC NUCLEOTIDE ENHANCERS AND CALCIUM ANTAGONISTS. Mary B. Engler. UCSF, San Francisco, CA 94143-0610

The fish oil omega-3 fatty acids, eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), are associated with blood pressure lowering properties; however, the mechanisms(s) of action remain undefined. The vasodilator actions of EPA and DHA may contribute to such antihypertensive effects. Therefore, the vascular effects of DHA, in comparison to other known vasodilators, such as calcium antagonists (D600), cyclic GMP enhancers (sodium nitroprusside-SNP, nitroglycerin-NTG), and cyclic AMP enhancers (papaverine-PPV) were compared. Isometric force was measured and cumulative concentration (1-100μM) curves were generated in the isolated rat aortic ring preparation. DHA (1-100μM) evoked significant relaxation (15±2%-45±10%, $p<0.01$) in 10^{-6}M norepinephrine (NE)-contracted rings. The rank order of vasodilator effects in producing relaxation (%) is as follows: 1μM - SNP(92%)> NTG(36%)> D600(16%)> DHA(15%); 10μM - SNP(96%)> NTG(65%)> PPV(35%)> DHA(30%)> D600(28%); 100μM - PPV(120%)> SNP(103%)> NTG(95%)> D600(60%)> DHA(45%). The vasodilator profile of DHA is dissimilar to those vasodilators which act by mechanisms including cAMP, cGMP, and calcium antagonism. These results suggest that the mechanisms of vascular relaxation such as, elevation of cyclic nucleotides (SNP, NTG, PPV) and calcium antagonism of potential-operated channels (D600) are different from those induced by DHA.

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The number to the left of the decimal designates the session. The abstract number follows the decimal. A zero indicates that participation was invited and an abstract was not required.