W-AM-J7

FLUORESCENCE ANALYSIS OF THE TRANSCRIPTIONAL ACTIVATION DOMAIN OF THE HERPESVIRUS PROTEIN VP16 REVEALS A HIGHLY FLEXIBLE STRUCTURE. ((Fan Shen*, Steven J. Triezenberg*, Denise Porter#, Jay R. Knutson#, and Preston Hensley+)) *Dept. of Biochemistry, Michigan State University, East Lansing MI 48824-1319, #NHLBI, LCB, NIH, Bethesda MD 20892, and +Dept. of Macromolecular Sciences, SmithKline Beecham, King of Prussia PA 19406-0939.

VP16, a virion protein of herpes simplex virus, specifically and potently activates transcription of viral immediate early genes. The transcriptional activation function has been mapped to the C-terminal 78 amino acids (residues 413-490). Extensive mutational analysis has shown that this function can be diminished or abolished by subtle amino acid substitutions in this domain. Biochemical analyses suggest that VP16 activates transcription by interacting with TFIID, TFIIB, or other components of the basal transcriptional machinery through contact with the activation domain. However, little is known about the structure of the domain or the mechanism by which it interacts with these proteins. Here, we describe a fluorescence analysis employing chimeric proteins comprising the DNA-binding domain of GAL4 (1-147) fused to the activation domain of VP16 (413-490 or subdomains thereof). Trp36 of GAL4 was replaced by Val and Trp residues were substituted for Phe at either 442 or 473 of VP16, with only modest effects on activity, thus obtaining unique fluorescence probes at two positions in the activation domain. The results of dynamic quenching, DAS, and time-resolved anisotropy studies show that the Trp residues at either position have very similar fluorescence properties, are highly mobile and are very solvent exposed. These results are not predicted by mutational studies, which suggest that the activation function requires some specific structure. These data are compatible with published CD and NMR analyses of peptides containing the VP16 activation domain. Together, these data suggest that a significant structural transition in the activation domain must accompany interaction of VP16 with target protein(s) in the basal transcription machinery.

W-AM-J8

AN *IN VITRO* MODEL OF CARDIAC MYOCYTE HYPERTROPHY DISPLAYS DOWN-REGULATION OF THE MESSENGER RNAs (MRNAs) ENCODING CA^{2+}-CYCLING PROTEINS. ((S.A. Fisher, M. Absher, M. Periasamy, N.R. Alpert, and A.S. Rovner)) Department of Molecular Physiology and Biophysics, University of Vermont College of Medicine, Burlington, VT 05405.

The expression of mRNAs encoding the sarcoplasmic reticular proteins involved in Ca^{2+} cycling decrease both in animal models of pressure overload hypertrophy and in human heart failure. To test the hypothesis that increased levels of circulating catecholamines present in these conditions mediate such changes in gene expression, we have implemented a cell culture model of cardiac myocyte hypertrophy. Rat neonatal cardiocytes were isolated by serial enzyme digestion, and cultured in the presence or absence of 2 μM norepinephrine (NE) in a defined, serum-free medium. In some experiments, thyroid hormone (T3) was also included in the medium. Hypertrophy was assessed by measuring the amount of protein per cell, and the levels of the mRNA for calsequestrin (CSQ), phospholamban (PL) and the cardiac/slow muscle sarco-endoplasmic reticulum Ca^{2+}-ATPase isoform (SERCA2) were assessed by northern blotting and normalization to the levels of glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. NE caused a substantial increase in the amount of protein per cell irrespective of T3 status. However, in T3-containing cultures, treatment with NE produced a 30-50% decrease in the steady-state levels of SERCA 2, CSQ and PL mRNA. This result indicates that NE may modulate T3-sensitive gene expression in cardiac muscle cells, and suggests a mechanism for the down-regulation of Ca^{2+} cycling proteins in heart failure. (Supported by NIH P01 HL28001-11).

PROTEIN FOLDING: RECOGNITION OF SUBMOLECULAR DOMAINS

W-PM-Sym-1

PROTEIN MOTIFS AND FOLDING RECOGNITION. ((J.M. Thornton[1], C.M. Orengo[1], D.T. Jones[1,2], W.R. Taylor[2])) [1]Biomolecular Structure and Modelling Unit, Biochemistry and Molecular Biology Dept., University College London. Gower Street, London WC1E 6BT, UK. [2]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

When a new protein structure is solved, it is becoming increasingly common to find that this structure closely resembles another entry in the Protein Structure Databank, (PDB) despite a lack of sequence or functional similarity. We have known for some time that there is a limited number of secondary and supersecondary motifs (eg. $\beta\beta$-hairpin, Greek Key, $\beta\alpha\beta$ unit) and it is now apparent that this translates into a limited number of protein folds. We have systematically identified structural similarities in the PDB and classified the folds into families (1). In the $\beta+\alpha$ family of structures we find that two motifs (the split $\beta\alpha\beta$ unit and the $\beta\beta\beta\alpha$ meander) are very common (2). In the universe of proteins, the structures segregate into 3 groups (all α, all β and $\alpha\beta$), but some topologies recur much more frequently than expected (eg. the TIM barrels). We have been able to use the current growth in the database, combined with a knowledge of which folds recur, to estimate the total number of folds. Furthermore, the fold library we derive, provides the target structures for fold recognition from amino acid sequence, using the optimal sequence threading algorithm combined with empirical potentials (3). Recent results will be described.

1. Orengo C.M. et. al. (1993) Protein Eng. 6 485-500
2. Orengo C.M. et. al. (1993) Structure. 1 105-120
3. Jones D.T. et. al. (1992) Nature. 358 86-89

W-PM-Sym-2

THE SLOW EXCHANGE CORE AS THE PROTEIN FOLDING CORE. **((Clare Woodward)) Dept. of Biochemistry, University of Minnesota, St. Paul, MN 55108.**

The slow exchange core of a native protein contains the last 3-8 peptide NH hydrogens to undergo isotope exchange with solvent. It consists of segments of secondary structure packed by hydrophobic groups. The slow exchange core is highly correlated with sequences containing NH groups that are protected first during folding, and with those that are slowly exchanging in partially folded proteins. The correlation of the slow exchange core to the folding core suggests an insight into the central logic of protein structure and folding (Kim et al., *Biochemistry* 32, 9600-9608; Woodward, Cur. Op. Str. Biol. in press). Structure implies process; in this case, native state dynamic structure reflects initial events in folding, or, rigid native structure predicts function in folding. The region that is least flexible by H-exchange criteria apparently contains the noncontiguous segments involved in early folding interactions. The proposal is that the slow exchange core is *the* hydrophobic core, or essential tertiary kernel, containing the sequences encoding the fold. (Large proteins may have more than one.) Peptides with sequences corresponding to the core are expected to have a native-like fold. Partially folded proteins (molten globules, A states) are expected to be collapsed in the region of the slow exchange core. These points are illustrated by the folding behavior of BPTI having only one intact disulfide bond, 14-38.

W-PM-Sym-3

PROTEIN FOLDING: DRIVING FORCES AND THE LEVINTHAL PARADOX. ((Ken A. Dill, Kai Yue, and Klaus Fiebig)) University of California, San Francisco, CA 94143-1204

What forces drive the compactness, the symmetries and internal architectures, and the uniqueness, cooperativity, and folding kinetics of globular proteins? One view holds that proteins are folded by hydrogen bonding and helical tendencies of peptide bonds. We have explored an alternative view that the properties of globular proteins are mainly encoded in their sequences of hydrophobic and polar amino acids. We have recently found that some tertiary structural symmetries ("designable sequences") arise from sequences of nonpolar and polar amino acids that can fold to the fewest incorrect alternatives. The Levinthal paradox is the question of how a protein can find its unique native conformation without endless searching of its enormous conformational space. We believe proteins fold as "hydrophobic zippers."

W-PM-Sym-4

PROTEINS IN PIECES. ((J. CAREY)) Chemistry Dept., Princeton University, Princeton NJ 08544-1009.

Proteolytic dissection of native *trp* repressor and horse heart cytochrome c has been used to infer some of the steps in the folding pathways of the intact proteins. For both proteins, small fragments are capable of undergoing spontaneous noncovalent association to form subdomains with native-like secondary and/or tertiary structural features, suggesting that dissection/reassembly may be a general method to gain insight into the structures of folding intermediates. The importance of this approach is its simplicity and potential applicability to studying the folding pathways of a wide range of proteins. The proteases report on the structure and dynamics of the native state, circumventing the need for prior knowledge of the structures of folding intermediates. The observation that small fragments of proteins can associate noncovalently suggests that protein folding can be viewed as an intramolecular "recognition" process. The results imply that substantial information about protein structure and folding is encoded at the level of subdomains, and that chain connectivity has only a minor role in determining the fold.

W-PM-A1

THE TYROSINKINASE (TK) INHIBITOR, GENISTEIN, UNCOUPLES FORCE AND MYOSIN LIGHT CHAIN PHOSPHORYLATION (MLC-P) IN ILEAL SMOOTH MUSCLE.

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Protein tyr-phosphorylation may participate in mechansims which control agonist induced Ca^{2+}-sensitization of smooth muscle myofilaments through influencing MLC-P dependent and independent mechansims. In α-toxin permeabilized ileal longitudinal smooth muscle, force was increased by carbachol (10 μM, 10 μM GTP) at constant pCa 6.57 which was associated with 30% MLC-P at the peak of the contraction. In the presence of genistein (20 μg/ml), fibers remained nearly completely relaxed under the above activating conditions whereas MLC-P increased to 20%. This compares to 5% MLC-P of fibers relaxed at pCa 8. Several proteins are tyr-phosphorylated during activation by Ca^{2+} or carbachol which is also inhibited by genistein. One of the substrates has a M_r of 205 kDa in 5-20% gradient SDS-PAGE. This band is recognized by specific smooth muscle myosin heavy chain (MHC) antibodies obtained from Dr. Gröschel-Stewart. In high resolution SDS-PAGE (4%), the two isoforms of MHC were separated and identified with the MHC antibodies. Both these bands were also recognized with anti-phosphotyrosine antibodies suggesting that MHC may be one of the substrates of TK. Our results suggest tyr-phosphorylation may be involved in regulating the level of MLC-P but may also influence additional regulatory mechansim(s) which control the coupling between force and MLC-P.

Supported by the DFG (G.P.) and the Edwin Eddy foundation (J.D.S.).

W-PM-A2

KINETIC MECHANISMS OF TA-CALMODULIN INTERACTIONS WITH SMOOTH MUSCLE MLCK AND DERIVED PEPTIDES. ((K. Török and D.R. Trentham)) *NIMR, Mill Hill, London NW7 1AA, UK*

(Supported by NIH Grant HLB 15835 to PMI and MRC,UK)

Analyses of the interactions of 2-chloro-(ε-amino-Lys_{75})-(6-(4-diethylaminophenyl)-1,3,5-triazin-4-yl)-calmodulin (TA-calmodulin) with MLCK and derived peptides by fluorescence stopped-flow in 50 mM MES adjusted to pH 7.0 with KOH, 100 mM KCl, 2 mM $MgCl_2$ and 100 μM $CaCl_2$ at 21°C were consistent with the model:

$$\text{TA-cal} + \text{P} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{TA-cal.P} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{TA-cal.P*}$$

P	K_d	k_{+1}	$k_{+2}+k_{-2}$	k_{+2}	k_{-2}	k_{-1}
	nM	$M^{-1}s^{-1}$	s^{-1}	s^{-1}	s^{-1}	s^{-1}
Tyr-peptide	4.3	8.3×10^8	1.0	0.36	0.64	5.7
Trp-peptide	0.0096	8.2×10^8	1.0	*	*	*
MLCK	0.56	1.0×10^8	1.3	1.15	0.15	0.49

**Monophasic dissociation of binary complex, so* $k_{-1}k_{-2}/(k_{+2}+k_{-2})= 0.008$ s^{-1}. *Determination of* k_{-1}, k_{+2} and k_{-2} *requires measurement of* [TA-cal.P]/[TA-cal.P*].

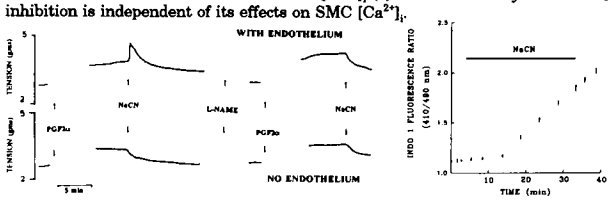
TA-calmodulin (a specifically labeled intensely fluorescent calmodulin with λ_{ex} 365 nm, λ_{em} 410 nm), Tyr-peptide (Ac-RRKYQKTGHAVRAIGRL-amide) and Trp-peptide (W_4 substituted Tyr-peptide) were > 98% pure. MLCK although chromatographically homogeneous, cannot be claimed a single molecular species. TA-calmodulin is a competitive inhibitor of MLCK with respect to calmodulin with K_i/K_m =3.6.

W-PM-A3

INHIBITION OF OXIDATIVE METABOLISM INHIBITS BASAL EDRF/NO PRODUCTION AND RELAXES VASCULAR SMOOTH MUSCLE IN A Ca^{2+}-INDEPENDENT MANNER

((Roy C. Ziegelstein, Sanford J. Gips, Pamela Ouyang, Thomas Aversano, Gemin Zheng, Howard S. Silverman)) Division of Cardiology, Johns Hopkins University and Francis Scott Key Medical Center, Baltimore MD 21224 (Spon. by F.C.P. Yin)

EDRF/NO production is dependent on oxidative metabolism (OM). To determine the effect of inhibition of OM on both endothelium-dependent (E+) and -independent (E-) vascular tone, isolated rat aortic rings were exposed to glucose-free 5% CO_2/HCO_3 buffer with 2 mM NaCN to inhibit OM. In PGF2α-preconstricted rings (bottom left) NaCN produced a transient E+ vasoconstriction (n = 11) which was abolished by pretreatment with the inhibitor of NO synthesis L-NAME (n = 3) and an E- vasodilation (n = 9). In isolated, indo 1 loaded dog coronary artery smooth muscle cells (SMCs), NaCN rapidly increased cytosolic Ca^{2+} ($[Ca^{2+}]_i$, bottom right), without changing cell length (n = 3). Thus, (1) basal EDRF/NO release by aortic rings is dependent on OM (2) inhibition of OM produces an E- relaxation of arterial rings (3) inhibition of OM increases SMC $[Ca^{2+}]_i$ (4) the vasodilatory effect of OM inhibition is independent of its effects on SMC $[Ca^{2+}]_i$.



W-PM-A4

EFFECTS OF PURINERGIC AGONISTS ON MUSCLE FIBRES FROM HYPERTROPHIED RAT URINARY BLADDER

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Tension responses induced by the purinergic agonists ATP and the stable ATP-analogue α-β-methylene-ATP were investigated in isolated muscle strips from normal and hypertrophic urinary bladders from the rat. Hypertrophy was induced by a partial ligation of the urethra giving an increase in bladder weight from about 65 mg to 285 mg. Activation with ATP and α-β-methylene ATP caused phasic, concentration-dependent, contractions. The sensitivity to ATP was about 100-fold lower than that for α-β-methylene ATP. The force of the contractions induced by the purinergic agonists was significantly lower in the hypertrophied bladder compared to the controls. The kinetics of the ATP induced responses was studied by photolytic release of ATP from caged-ATP in intact fibre bundles. The rate of contraction following photolytic release of ATP was slower, and the force amplitude lower, in the hypertrophic preparations compared to the controls. The results suggest changes in the purinergic receptor function or in the responses of the contractile system to transient increases in intracellular Ca^{2+} in the hypertrophic bladder.

W-PM-A5

EFFECTS OF BATH OSMOLARITY ON WHOLE CELL Ba^{2+} CURRENT, Ca^{2+}-DEPENDENT FORCE AND MYOGENIC TONE IN RAT CEREBRAL ARTERY: A COMMON MECHANISM?

((P.D. Langton and [1]W.R. Dunn)) Ion Channel Group, Dept. Cell Physiology and Pharmacology, University of Leicester, U.K. [1]Dept. Physiology and Pharmacology, University of Nottingham, U.K.

We have found that dihydropyridine (DHP) sensitive Ba^{2+} current in rat basilar artery myocytes is sensitive to bath osmolarity (control 292; hyposmotic 238; hyperosmotic 354 mosmol l^{-1}). Using amphotericin-B perforated patch whole cell recording, peak inward was -78pA (±17, 9) in control, increasing to -125pA (±8, 5) and declining to -40pA (±6, 4) during hypo- and hyper-osmotic superfusion, respectively (mean (±S.E., n)). These effects were reversible and were not due to changes in (or induction of) other conductances, or changes in capacitive surface area. Similar effects in response to positive and negative pressure applied to the patch pipette (conventional whole-cell) suggest the involvement of changes in cell volume (membrane stretch). Because of the sensitivity of arterial tone to DHP-channel antagonists we examined the effect of osmotic strength on force in intact cerebral vessels.

Firstly, sections of rat basilar artery were mounted in a wire myograph for measurement of isometric force. Relative steady-state force developed in response to high K^+ (40mM) was potentiated to 1.43±0.043 and depressed to 0.46±0.021 of control values by hypo- and hyper-osmotic solutions, respectively (n=14 tissues). Secondly, sections of rat middle cerebral artery were mounted in a Halpern-pressure myograph and pressurized to 70 mmHg, whereupon they developed myogenic tone (reducing mean dia. by 57±12μm from 232±6μm, n=13). Hypo- and hyper-osmotic bath superfusion resulted in 26μm (±4, 4) constriction and 25μm (±8, 5) dilation, respectively. These effects on force and diameter were reversible.

These data are consistent with the idea that osmotically induced changes in Ca^{2+} channel activity may also occur in intact tissue and may participate in the transduction of transmural pressure into the myogenic response.

Supported by the British Heart Foundation.

W-PM-A6

REGULATION OF CYCLIC (AND NON-CYCLIC) ADP-RIBOSE LEVELS IN HEART AND SMOOTH MUSCLE.

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Cyclic ADP-ribose (cADPR) has been found to activate the cardiac (but not the skeletal) type ryanodine receptor Ca^{2+} channel [Mészáros et al. Nature, 364: 76, 1993], while ADP-ribose (ADPR) was found to inhibit the channel (to be published). To consider these NAD metabolites as endogeneous regulators of the non-skeletal type ryanodine receptor channels in mammalian cells, a set of criteria must be met: first, there should be enzymatic systems present, which are capable of efficiently producing and eliminating ADPR and cADPR and, second, these systems should be under the control of signals of extracellular origin.

Here we present data which indicate that the first criterion is fullfilled in both cardiac and smooth muscle cells: 1) The production of both cADPR and ADPR were catalyzed by enzyme(s) that belong(s) to the family of NADase as evidenced by i) the HPLC analysis of the radio-labelled enzymatic products and ii) the sensitivity of the producing enzyme to 3-amino-benzamide (ABA). 2) The elimination of cADPR was also mediated by an ABA-sensitive NADase, yielding ADPR as product, while ADPR was degraded into adenine by an unknown enzyme. The rate of adenine formation was about the same in both cardiac and smooth muscles, while the formation rate for both cADPR and ADPR was 5-10-fold higher in smooth than in cardiac muscle. Kinetic analysis as well as autoradiography suggests that the immediate trigger for cADPR production is the (auto)ADP-ribosylation of a membrane-bound NADase. These results indicate that the enzymatic machinery to control cADPR and ADPR levels in cardiac and smooth muscle are operational. [Supported by the American Heart Assiciation, GA Affiliate.]

W-PM-A7

INTRACELLULAR Ca^{2+} TRANSIENTS INDUCED BY HIGH EXTERNAL K^+ AND TETRACAINE IN CULTURED RAT MYOTUBES

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Cultured myotubes from rat neonatal skeletal muscle were used to measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane potentials using the indo-1 microfluorimetry method and the nystatin perforated patch technique. Sudden increases in external $[K^+]_o$ from 5 mM to either 22, 42 or 84 mM elicited transient elevations in $[Ca^{2+}]i$ from a resting level of 87.8 ± 11 (n = 27) to peak values of 297, 409 and 454 nM, respectively. The complex Ca^{2+} release response induced by elevated $[K^+]_o$ can be described by a minimal empirical model involving two components with different activation-inactivation kinetics. Membrane potential changes induced by elevated $[K^+]_o$ followed the Nernst equation for $[K^+]_o$. While the fast transitory component was rather insensitive to $[Ca^{2+}]_o$ and nifedipine, the slow component was inhibited by the dihydropyridine (10 μM) in a Ca^{2+} deficient medium. We also found that tetracaine (0.05 to 2 mM) elicited a fast elevation in $[Ca^{2+}]_i$ followed by a rise at a constant rate to levels as high as 1-2 μM. Tetracaine evoked $[Ca^{2+}]_i$ rise was readily reversible. Simultaneous measurements of the membrane potential and the $[Ca^{2+}]_i$ rise suggest that the fast component is coupled to a rapid depolarization of the membrane induced by the anesthetic.

Partly financed by MDA, Fondecyt 9311089 and DTI B3320.

W-PM-A8

EFFECTS OF EXTRACELLULAR HEPARIN ON EXCITATION-CONTRACTION COUPLING IN FROG SKELETAL MUSCLE FIBERS.

((M. Martinez, J.M. Farias, M.C. Garcia and J.A. Sanchez)). Department of Pharmacology, CINVESTAV-IPN, A. P. 14-740, Mexico D.F. 07000.

Heparin binds with high affinity to the DHP receptor of skeletal muscle (Knaus et al. JBC, 265:156, 1990). Our experiments investigate the action of heparin on isometric tension, Ca signals, DHP-sensitive Ca currents (I_{Ca}) and charge movement. **Methods:** 1.- Single muscle fibers from frog (*Rana montezumae*). Cells were loaded with AM-Rhod 2 (10 μm) and fluorescence signals were obtained with a photodiode. Tension was simultaneously measured. 2.- Voltage-clamp experiments in cut fibers using the triple Vaseline gap technique. **Results:** Heparin (100 μg/ml) reversibly potentiates twitch, tetanic tension and Ca signals by ca. 50%. Heparin blocks I_{Ca} by 30% and shifts the voltage-current relation towards more negative potentials by 15 mV. Heparin makes activation more rapid at all potentials and slows down the deactivation during tail currents. Double pulse experiments indicate that the kinetics of I_{Ca} is dependent on the frequency of stimulation as shown by others. Heparin further accelerates this process. Half-time of activation increases by 13 ms during the second pulse in control experiments and by 22 ms in the presence of heparin. These results suggest that heparin shows some similarities with lyotropic anions on E-C coupling and that the potentiating effect of heparin may be related to the kinetic effects on Ca channels in skeletal muscle.

W-PM-A9

CALCIUM-DEPENDENT CHLORIDE CURRENT IN FROG SKELETAL MUSCLE.

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Nonlinear membrane ionic current was studied in stretched cut frog twitch fibers (sarcomere length ~4 μm) with the double Vaseline-gap voltage clamp technique. After the Na^+ and K^+ currents were suppressed, a delayed outward ionic current having a time course similar to that of the delayed rectifier current was observed on depolarization. This current was not affected by the application of 1 mM 3,4-DAP to the external solution nor by the introduction of K^+ or Na^+ to the internal solution, suggesting that the current was not carried by cations flowing through the delayed rectifiers. On the other hand, it was greatly reduced when the Cl^- in the external solution was completely replaced by methanesulphonate, sulphate, glutamate or gluconate, or by applying 9-ACA to the external solution, indicating strongly that it was carried by Cl^- flowing through anion channels. It was also partially reduced by blockers of Ca-dependent Cl channels, such as SITS and furosemide, in a dose-dependent and a voltage-dependent manner, suggesting that part of the Cl current in the muscle fibers could be Ca-dependent, which was further supported by the observation that a portion of the current was suppressed when 20 mM EGTA was applied to the internal solution. In a group of fibers, the total Cl current was separated into a Ca-dependent and a Ca-independent component. Each component accounted for approximately half of the total Cl current. (Supported by NIH NS-21955 and a grant from MDA).

W-PM-A10

CALCIUM TRANSIENTS ASSOCIATED WITH THE T-TYPE CALCIUM CURRENT IN MYOTUBES.

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Embryonic muscle expresses a T-type calcium current (also termed I_{fast}), but the contribution of this current to changes in myoplasmic calcium concentration is not known. We have examined calcium transients (ΔCa) caused by the T-type current in both normal myotubes (which also express a DHP-sensitive current, I_{slow}) and in dysgenic myotubes which lack I_{slow} but variably display another DHP-sensitive current (I_{dys}). In dysgenic myotubes expressing only I_{fast}, depolarization elicited a small ΔCa whose amplitude as a function of test potential mirrored that of I_{fast}; also, the time course of ΔCa followed the integral of the calcium current. A similar relationship between ΔCa and current was found in dysgenic myotubes injected with cDNA for the cardiac DHP-sensitive channel and in normal myotubes at low voltages that activated T current. However, ΔCa in normal myotubes at high voltages did not follow the integral of I_{slow}. At -20 mV, which activated peak I_{fast}, ΔCa measured at the end of the 15-ms test pulse was an average of ~4% (n=17) of the ΔCa measured at test potentials ≥20 mV, where I_{fast} was small and I_{slow} was large. Addition of 0.3-1.0 mM amiloride, which reduced I_{fast} without affecting I_{slow} or I_{dys}, caused a reduction of ΔCa associated with I_{fast}. Supported by grants from NIH (NS 24444 and NS 28323 to K.B.) and AHA of Colorado (to J.G.).

Na CHANNEL GATING AND REGULATION

W-PM-B1

VOLTAGE-DEPENDENT REGULATION OF MODAL GATING IN THE RAT SkM1 SODIUM CHANNEL EXPRESSED IN *XENOPUS* OOCYTES.

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The TTX-sensitive rat skeletal muscle sodium channel (rSkM1) exhibits two modes of inactivation (fast vs. slow) when the α subunit is expressed alone in *Xenopus* oocytes. In this study, two components are found in the voltage-dependence of "steady-state" current inactivation, one having a $V_{\frac{1}{2}}$ in the expected voltage range (~-50 mV, I_N) and the other with a more hyperpolarized $V_{\frac{1}{2}}$ (~-130 mV, I_H). The I_N component is associated with the gating mode having rapid inactivation and recovery from inactivation of the macroscopic current, while I_H corresponds to the slow inactivation and recovery mode. These two components are interconvertible and their relative contribution to the total current varies with the holding conditions: I_N is favored by prolonged hyperpolarization. When the rat sodium channel β_1-subunit was co-injected with rSkM1, I_H was essentially eliminated and the inactivation kinetics of macroscopic current became fast. It appears that these two current components and their associated gating modes may represent two conformations of the α subunit, one of which can be stabilized either by hyperpolarization or by association with the β_1 subunit.

W-PM-B2

β_1 SUBUNIT MODULATION OF Na CHANNEL INACTIVATION DOES NOT OCCUR FROM THE CYTOPLASMIC SIDE.

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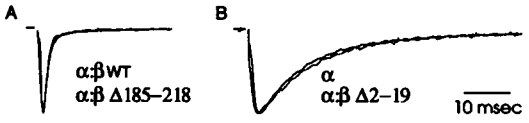
Injection of mRNA encoding the α subunit of the rat skeletal muscle Na channel into *Xenopus* oocytes results in a Na current which decays with a τ of 9-12 ms. Co-expression of the Na channel β_1 subunit from human brain accelerates the inactivation of the current at least 5 fold. To further understand the mechanisms by which the β_1 subunit modifies Na channel inactivation, deletion mutations were made. Deletion of the entire cytoplasmic tail did not significantly affect β_1 subunit function. Superposition of Na current recorded from a cell co-injected with α and β_1 wildtype showed little difference in macroscopic inactivation (Fig A). In contrast, deletion of the region of the β_1 subunit encoding the putative signal peptide completely eliminated its modulation of the Na current inactivation. Figure B superimposes current recorded from a cell injected with α subunit alone with that from a cell injected with α and β_1 mutant containing deletion of the signal peptide. Both currents decayed slowly (τ of 10 ms). Finally, deletion mutations of the putative extracellular region were found to disrupt the function of the β_1 subunit. Our results suggest that the β_1 subunit may be interacting with the α subunit via an extracellular site.



W-PM-B3

PROTEIN KINASE A MODULATES CARDIAC SODIUM CHANNELS EXPRESSED IN *XENOPUS LAEVIS* OOCYTES.

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Adrenaline and acetylcholine act stimulatory and inhibitory on heart and activate differentially cAMP and Ca^{2+}-dependent protein kinases (PKA and PKC) via β_2-adrenergic (β_2AR) and muscarinergic M_2-receptors. We investigated functional effects of PKA stimulation by heterologous expression of cardiac sodium channel α_{SkM2} and β-subunits, G-protein α-subunits and β_2-adrenergic receptors in frog oocytes. We have observed that sodium currents (I_p) from cardiac α_{SkM2} with and without the β-subunit were enhanced upon (i) cytosolic injection of cAMP and by (ii) extracellular application of 10^{-5}M isoproterenol. Isoproterenol applied after cAMP injection did not result in further enhancement of I_p. cAMP did not effect kinetic parameters but increased only maximal sodium conductance and did not depend on intracellular calcium. Interestingly, coexpression of the G_s α-subunit reduces the effect of β_2AR stimulation, whereas the G_{i2} α-subunit enhances it, suggesting some basal activity of these proteins, when overexpressed in oocytes. Mutation of the five major consensi sites for PKA phosphorylation (different from the sites existing in α_{IIA}) did not abolish I_p enhancement.

Our data demonstrate for the first time differential modulation of sodium channel isoforms by PKA in the same cellular environment, i.e. downregulation of α_{IIA} and upregulation of α_{SkM2} as well as differential modulation of α_{SkM2} by PKA and PKC (this issue).

W-PM-B4

PROTEIN KINASE A PHOSPHORYLATION OF THE RAT BRAIN IIA SODIUM CHANNEL IS NOT ESSENTIAL FOR NORMAL FUNCTION IN XENOPUS OOCYTES. ((R.D. Smith and A.L. Goldin)) Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717-4025.

The voltage-gated sodium channel from rat brain contains five protein kinase A (PKA) phosphorylation sites in the cytoplasmic linker between domains I and II. We have constructed a mutation of the rat brain IIA sodium channel in which all of the consensus PKA sites have been deleted, and one in which all of the potentially phosphorylated serine residues have been replaced by alanines. Both of these mutants are functional when expressed in *Xenopus* oocytes, with electrophysiological properties similar to those observed for the wild-type channel. To determine if the PKA sites are normally phosphorylated, we have examined the channels by immunoprecipitation. To increase specificity, a synthetic epitope termed FLAG was incorporated into the amino terminal region of the channel, and monoclonal antibodies directed against the FLAG epitope were used for immunoprecipitation. Insertion of the epitope into the amino terminal region did not alter the electrophysiological properties of the channels. After labeling with ^{35}S-methionine, a diffuse band corresponding to a protein between 250-300 kd was detected from *Xenopus* oocytes injected with in vitro transcribed RNA. This protein was phosphorylated in vivo, as shown by labeling with γ-^{32}P-ATP. The mutant channels in which the PKA sites in the I-II linker were eliminated demonstrated reduced incorporation of ^{32}P, indicating that these sites are normally phosphorylated. These results indicate that the rat brain IIA sodium channel is phosphorylated by protein kinase A in *Xenopus* oocytes, but that this phosphorylation is not essential for normal electrophysiological function.

W-PM-B5

ACTIN PHOSPHORYLATION BY PROTEIN KINASES A AND C EFFECTS EPITHELIAL SODIUM CHANNEL REGULATION. ((A.G. Prat and H.F. Cantiello)). Renal Unit, Massachusetts Gral. Hosp., Charlestown, MA 02129, and Dept. Med., Harvard Med. Sch.

Apical epithelial Na^+ channel activity is regulated by two important second messenger mechanisms: the cAMP/PKA- and the PKC-mediated pathways. The actual substrate(s) of phosphorylation by either kinase remain unknown. We recently demonstrated that changes in actin filament organization regulate Na^+ channel activity in A6 renal epithelial cells. We were therefore interested to determine whether the phosphorylation of actin by protein kinases A or C may have differential effects on actin organization and, in a consequence, Na^+ channel activity. Addition of PKC to excised patches of A6 cells with spontaneous Na^+ channel activity inhibited ion channel activity. Addition of PKA to patches with no apparent channel activity in contrast, elicited Na^+ channel activation. To determine whether actin phosphorylation modifies its ability to polymerize, actin was incubated in the presence of PKA or PKC and ATP. The PKA- or PKC-phosphorylated actin were allowed to polymerize and then analyzed by gel electrophoresis. PKA-phosphorylated actin showed a lower ability to polymerize compared to native actin. The PKC-phosphorylated actin in contrast, completely failed to polymerize. PKA-phosphorylated actin filaments activated Na^+ channel activity (n=9). In contrast, the addition of PKC to excised patches containing Na^+ channels previously activated by actin, completely inhibited Na^+ channel activity (n=3), an effect similar to that observed with DNAse I which prevents actin polymerization. The results indicate that the selective phosphorylation of actin by various protein kinases may represent a novel regulatory mechanism for Na^+ channel regulation.

W-PM-B6

ANTISENSE β_1-DNA BLOCKS MODULATION OF μ1 Na CHANNELS BY BRAIN mRNA

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Functional Na channels are expressed in *Xenopus* oocytes micro-injected with cRNA from cloned brain (RIIA) and adult skeletal muscle (μ1) α-subunits alone. Such channels spend a large percentage (50-95%) of their time in anomalous, slow-inactivating (mode 2) gating states (Zhou et al., (1991) **Neuron, 7**, 775-785.). When co-expressed with small amounts of RNA from (a) muscle or brain tissue, (b) a brain cDNA library, or (c) a cloned brain (or muscle) β_1 Na channel subunit cDNA, gating is strongly shifted towards rapidly inactivating states (modes 1A/1B). We observed that anti-sense oligonucleotides derived from the β_1-ORF suppressed the modulation of both RIIA and μ1-channel gating by (b and c), indicating that β_1 transcripts were both necessary and sufficient to produce modulation by the brain cDNA library. Experiments are being conducted to test whether modulation requires co-translational assembly of α and β_1 subunits. We further note that Na channels expressed in mammalian cells from μ1 and RIIA α-subunits alone exibit predominantly (95-97%) fast-inactivating states in the absense of exogenous β_1-subunit cDNA (Ukomadu et al.,(1992) **Neuron, 8**, 663-676). The current amplitudes for these channel isoforms was decreased and inactivation kinetics may be speeded (μ1) or slowed (RIIA) by activators of second messenger associated Protein kinase A and Protein Kinase C. These results suggest that association with β_1 subunits and the state of protein phosphorylation may affect the distribution between intrinsic gating states of muscle and neuronal isoforms.

W-PM-B7

THE SINGLE CHANNEL BASIS FOR SLOW INACTIVATION OF Na^+ CHANNELS. ((R.L. Ruff)) Cleveland VAMC, Case Western Reserve Univ., Cleveland, OH 44106

Mammalian skeletal muscle has two distinct inactivation processes. Fast inactivation (FI) closes channels on a msec time scale, whereas slow inactivation (SI) takes minutes. FI terminates the action potential. SI is too slow to affect action potential termination. However, SI operates at more negative potentials than FI, so that SI regulates the Na^+ current as a function of the membrane potential. On a single channel level, SI could reduce I_{Na} by decreasing single channel conductance or by decreasing the number of excitable channels. We recorded macroscopic I_{Na} with a loose patch voltage clamp and single channel currents were measured simultaneously on an adjacent region of membrane from the same rat skeletal muscle fiber. The holding potentials of the two clamps were kept the same. This enabled us to monitor simultaneously the impact of SI on macroscopic and single channel currents. Changes in the macroscopic currents due to SI were accompanied by changes in the maximum number of excitable channels within a patch of membrane. The single channel current amplitudes did not change during the development of and recovery from SI. Therefore, SI results from Na^+ channels accumulating in a non-conducting state. Several different classes of Markovian kinetic models have been proposed to describe SI of Na^+ channels. Major distinctions among the models are whether the SI state is accessed through only the: open, FI or closed channel state. Using different pulse protocols we determined that channels do not have to transit through the open or FI states to access the SI state. The data was most consistent with models in which the SI state can be directly accessed from both the closed and FI states. [Supported by the Office of Research and Development, Medical Research Service of the Department of Veterans Affairs]

W-PM-B8

STATIONARITY OF SODIUM CHANNEL GATING KINETICS IN INSIDE-OUT EXCISED PATCHES FROM NEUROBLASTOMA N1E 115. ((L. Goldman)) Dept. of Physiology, School of Medicine, Univ. of Maryland, Baltimore, MD 21201. (Spon. by J.O. Bustamante)

Channel gating parameters are reported to shift along the voltage axis on patch excision (see e.g. Fenwick et al. (1982) J. Physiol., 331:599). It is not clear whether kinetic parameters shift rapidly on excision and then remain stationary (Kunze et al. (1985) J. Gen. Physiol., 86:691) or continue to shift. To assess stationarity in neuroblastoma N1E 115, 40 ms steps from a holding potential of -120mV to a test potential of -30 or -40 mV were presented, and ensemble average currents constructed from the single channel records. Test potential was constant for any individual patch. Capacitative currents were reduced by analog subtraction. Residual capacitative and leak currents were eliminated by digital subtraction of traces with no channel openings. Pipettes were sylgard coated. Ensembles were constructed at various times after excision and single exponentials fitted to the current decay. No recordings were made for the first 5 min. In one patch (-30 mV test, 7°C) ensembles were constructed at 20,40,60,90 and 120 min after excision. Fitted time constants were 4.62 (-1.7%), 4.33 (-7.9%), 5.08 (+8.1%), 4.45 (-5.3%) and 5.02 (+6.8%)ms, (numbers in parentheses indicate the % difference from the mean). Decay time constant is essentially unchanged over this interval. % difference from the mean time constant for each individual patch was plotted against time from excision for each of 8 patches. There was no obvious trend in the pooled data. Na channel kinetics in neuroblastoma remain stationary from at least 5 min to 2 hr following excision. Supported by N.I.H. grant NSO7734, a grant-in-aid from the American Heart Association, and the Bressler Research Fund, Sch. of Med., Univ. of Md.

W-PM-B9

TIME COURSE OF SODIUM CHANNEL INACTIVATION FROM CLOSED STATES AS DETERMINED BY SINGLE CHANNEL RECORDING. ((L. Goldman)) Dept. of Physiology, School of Medicine, Univ. of Maryland, Baltimore, MD 21201.

Na channels can inactivate without opening, but nearly nothing is known about this process. Inactivation from closed states can be studied with single channel recording and a conventional conditioning pulse–test pulse protocol. Ensemble average currents are constructed from the single channel recordings during the test pulses. Peak ensemble test pulse current against conditioning pulse duration describes the time course of inactivation during the conditioning pulse. By including in the ensemble only traces for which no channels opened during the conditioning pulse, inactivation from closed states is isolated (Aldrich and Stevens (1983) Cold Spring Harbor Symp. Quant. Biol., 48:147). Experiments were done on inside-out excised patches from neuroblastoma N1E 115. Channel kinetics are stationary over the 1 to 1.5 hr needed. Temperature was 7.5 ± 0.5°C. Inactivation from closed states develops as a single exponential. Mean time constants were 21.46ms at -60, 30.44ms at -70, and 64.93ms at -80mV. Steady state values were 0.027, 0.148, and 0.42 at -60, -70 and -80mV. In general, closed state inactivation should not proceed as a single exponential irrespective of which closed state or states directly inactivate. Computations with a two closed, one inactivated, and one conducting state model indicated that (for very negative holding potentials) closed state inactivation develops with an initial delay if the closed state adjacent to the conducting state directly inactivates, and with an initial faster relaxation if the closed state furthest from the conducting directly inactivates, suggesting that any additional components may be too small to resolve in this data set. Supported by N.I.H. grant NS07734, a grant-in-aid from the American Heart Association, and the Bressler Research Fund, Sch. of Med., Univ. of Md.

PHOTOSENSORY TRANSDUCTION AND PHOTOTAXIS

W-PM-C1

A CONTRIBUTION TO THE INTERPRETATION OF FLUENCE RESPONSE CURVES IN *HALOBACTERIUM HALOBIUM*: THE ROLE OF NOISE INHERENT IN CHEMICAL REACTIONS.
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Phobic responses to light stimuli in *H.h* have been studied aiming to determine the number of absorbed photons required to elicit a behavioural change (Marwan and Oesterhelt, 1988; Takahashi et al., 1992). In the former paper the statistic nature of the light stimulus is taken into account, using Poisson statistics for the interpretation of fluence-response curves. In the latter one even the motor switch is schematized as a probabilistic system, the probability of reversal depending on the concentration of a switching factor, which in turn depends on the number of photons actually absorbed. The noise inherent in chemical reactions, relevant when the number of involved molecules is not high, has not yet been taken into account in the interpretation of fluence-response curves. Here this is done through Montecarlo simulations of the kinetic models proposed (Marwan and Oesterhelt, 1987; Lucia et al., 1992; Takahashi et al, 1992) for the transduction chain in *H.h.*

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W-PM-C2

SPECTROSCOPIC AND BIOCHEMICAL IDENTIFICATION OF A PHOTOSENSITIVE RETINAL-PROTEIN IN LAMPREY PINEAL GLAND. ((K Parker, J. Lee, M. Jeffress, J.G. Fukushima, I. Szundi, and R.A. Bogomolni)) Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064

Daily light cycles entrain a biological clock that regulates the rythmic synthesis and release of the hormone melatonin in the pineal gland of vertebrates. In mammals, photic information gathered by photoreceptor cells of the lateral eyes is transmitted to the pineal gland via neural connections while in fish, amphibians, reptiles and birds, photoreception and light signal transduction take place in the pinealocyte cell. Using microspectrophotometry, retinal radiolabeling and SDS-page we have identified in the pineal complex of the juvenile California lamprey (*Entosphenus tridentata*) a photosensitive 31 kD retinal chromoprotein absorbing maximally at around 490 nm. This pineal-Rhodopsin pigment (p-Rho) is presumably the photoreceptor. P-Rho has a smaller molecular mass than all known visual rhodopsins, contains all-trans rather than 11-cis retinal as chromophore and photoconverts to a Metarhodopsin-like state that peaks at a shorter wavelength (λ_{max} < 370 nm) than the visual Metarhodopsin of the same organism (λ_{max}=380 nm).

W-PM-C3

THE ACTIVITIES OF RETINOIDS DURING FORMATION AND PHOTO-ACTIVATION OF VISUAL PIGMENTS. ((D.W. Corson and R.K. Crouch)) Medical University of South Carolina, Charleston, SC 29464

Over the last several years, we have been using natural and synthetic analogues of retinal to explore the physiological activity of retinoids in the relief of opsin desensitization at the time of pigment formation and in excitation at the time of pigment activation by light. Normally, 11-*cis* retinal restores sensitivity to bleached photoreceptor cells by relieving opsin desensitization and by increasing the quantity of photoactivatable pigment when it combines with opsin. When activated by the absorption of a photon, regenerated visual pigment then produces a normal quantal response in a dark-adapted cell. We have recently found that opsin desensitization, but not pigment depletion, can be relieved by 11-*cis*-locked retinoids in rods[1] and fragments of retinal as small as β-ionone in cones[2]. Some retinal analogues such as 4-hydroxy retinal[3] and 9-*cis* 13-desmethyl retinal (reported here) are capable of full participation in both relief of opsin desensitization and photoexcitation. Others such as 11-*cis* 9-desmethyl retinal[4] appear to be fully active in relieving opsin desensitization but participate to only a limited extent in photoexcitation. The nature of the specific interactions between these modified receptor ligands and individual amino acids of opsin remain to be determined. [1]Corson *et al.*, 1990, *PNAS 87*:6823-6827; [2] Jin *et al.*, 1993, *Neuron 11*:513-522; [3] Corson *et al.*, 1990, *Biophys. J. 57*:109-115; [4] Corson *et al.*, 1991, *Biophys. J. 59*:408a.

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W-PM-C4

SPECTROSCOPIC STUDIES OF A PHOTORECEPTOR MODEL SYSTEM ((F. Lenci, N. Angelini, F. Ghetti, A. Sgarbossa)) Istituto Biofisica CNR, Pisa, Italy, ((A. Vecli, C. Viappiani, A. Losi)) Dipartimento Fisica Universita', Parma, Italy.

In photoresponsive ciliates, like *Blepharisma japonicum* and *Stentor coeruleus*, the photoreceptor pigments responsible for photomotile reactions are hypericin-type chromophores packed in highly osmiophylic subpellicular granules. The photophysical properties and the photochemical reactivity of these clustered pigments are not known and can be quite peculiar. Liposomes loaded with hypericin can constitute a simple model system, appropriate for understanding the primary light-induced molecular events triggering the sensory chain in these microorganisms. Optical absorption, steady-state and time-resolved fluorescence, pulsed photoacustic calorimetry have been used to measure spectral distributions, fluorescence lifetimes, radiative and radiationless transitions quantum yields of hypericin, when assembled into liposomes made up of different phospholipids. With respect to hypericin ethanol solutions, both absorption and fluorescence maxima are 5-8 nm red shifted when the pigment is inserted into the lipidic microenvironment, regardless of the number N of hypericin molecules per liposome, whereas the relative fluorescence quantum yields and lifetimes decrease increasing N over 20. Measurements of radiationless transitions quantum yields in the different experimental conditions allow to calculate the energy balance for each model system.

W-PM-C5

REMOVAL OF TRANSDUCER HTRI ALLOWS ELECTROGENIC PROTON TRANSLOCATION BY SENSORY RHODOPSIN-I. ((R.A. Bogomolni[1], W. Stoeckenius[1], I. Szundi[1], E. Perozo[2], K. D. Olson[3], and J.L. Spudich[3])) [1]Department of Chemistry and Biochemistry, Univ. of California, Santa Cruz, CA 95064, [2]Jules Stein Eye Institute, UCLA, Los Angeles CA 90024, [3]Dept. of Microbiol. and Molec. Genetics, Univ. of Texas, Houston, TX 77030.

Sensory Rhodopsin-I (sR-I) expressed in cells devoid of HtrI has altered photochemical kinetics and proton diffusion pathways (see J.L. Spudich et al. abstract in this issue). In addition, light induces transmembrane electrogenic proton translocation in envelope vesicles prepared from HtrI-deficient, but not HtrI-containing, cells. Proton ionophores abolish the light-generated membrane potential (~100 mV, negative inside) and reduce the net light-induced acidification of the medium (5-6 H^+/sR-I) to about one H^+ per S_{373} accumulated in the photosteady state. Proton transport is maximal at neutral pH and is undetectable above pH 8.5 and below pH 6.0. The action spectrum for the light-induced electrical potential peaks at 540 nm, blue-shifted from the pigment's 590nm maximum. Similar shifts have been reported for phototactic responses of wild type cells under high intensity light and for maximal absorbance depletion of other HtrI-deficient preparations. We tentatively interpret this vectorial proton translocation as a proton leakage to the exterior medium through a path that is blocked in the presence of HtrI. (Supported by NIH grants GM-43561 and GM-27750)

W-PM-C6

A PROTON-CONDUCTING PATH IN SENSORY RHODOPSIN I IS BLOCKED BY TRANSDUCER INTERACTION. ((J.L. Spudich, K.D. Olson, E.N. Spudich, and V.J. Yao)) Dept of Micro & Molec Genet, Univ of Texas, Houston TX 77030.

Sensory rhodopsin I (SR-I λ_{max} 587 nm) is a phototaxis receptor controlling the flagellar motor of the archeon *Halobacterium salinarium*. The receptor resembles the visual pigments of higher organisms, consisting of a single polypeptide which folds into seven membrane-spanning α-helical segments forming an internal pocket where the chromophore retinal is bound. Photoisomerization of retinal in SR-I generates a signaling conformation of the receptor which is blue-shifted to λ_{max} 373 nm (species S_{373}). The S_{373} conformation transmits a signal to the membrane bound transducer protein HtrI. Several lines of evidence indicate photoconversion is accompanied by deprotonation of the Schiff base attachment site of the retinal chromophore buried in the photoactive center of the receptor. Using recombinant DNA methods we have expressed SR-I protein in its native *H. salinarium* membrane in the presence and absence of the transducer HtrI. We observe in transducer-free membranes that the formation of S_{373} releases protons (1 per S_{373} formed) and the first order rate constant for S_{373} reprotonation is proportional to bulk phase proton concentration. The presence of transducer blocks proton release and renders S_{373} decay pH-independent. HtrI deletion analysis localizes receptor/transducer interaction sites to that part of the transducer containing its transmembrane and cytoplasmic linker domains. REFERENCES: Spudich, E.N. & J.L. Spudich (1993) The photochemical reactions of sensory rhodopsin I are altered by its transducer. J. Biol. Chem. 268:16095-16097; Olson, K.D. & J.L. Spudich (1993) Removal of the transducer protein from sensory rhodopsin I exposes sites of proton release and uptake during the receptor photocycle. Biophysical J., *in press*.

W-PM-C7

HETERONUCLEAR MULTIDIMENSIONAL NMR STUDIES OF RECOVERIN: SEQUENTIAL ASSIGNMENTS AND SECONDARY STRUCTURE. ((J. Ames, L. Stryer & M. Ikura)) Dept. of Neurobiology, Stanford University, Stanford, CA 94025 and Ontario Cancer Institute, Univ. of Toronto, Toronto, Ontario, Canada. (Spon. J. Ames)

Recoverin, a calcium sensor in vision, is a recently discovered member of the EF-hand superfamily. The calcium-bound form of recoverin prolongs the lifetime of photoexcited rhodopsin. Retinal recoverin contains a covalently attached myristoyl or related fatty acyl group at its amino terminus. The binding of multiple Ca^{2+} induces the insertion of recoverin into disk membranes. The structure of recombinant unmyristoylated recoverin containing a single bound Ca^{2+} has recently been solved at 1.9 Å resolution by x-ray crystallography (Flaherty, Zozulya, Stryer, and McKay, *Cell*, 75, in press). We are carrying out NMR studies of myristoylated recoverin to learn how its calcium-myristoyl switch operates and to serve as a basis for studies of recoverin-target complexes. Recombinant myristoylated recoverin uniformly labeled with ^{15}N and ^{13}C was expressed in *E. coli*. Triple-resonance 3D-NMR experiments were performed to obtain sequential assignment of the backbone ^1H, ^{13}C and ^{15}N resonances. Sequence-specific assignments for ~80% of the backbone resonances have been obtained. Based on NOE, backbone amide proton exchange, $^3J_{NH\alpha}$ coupling constants and chemical shift data, we have determined most of the secondary structure of myristoylated recoverin deviod of Ca^{2+}. The positions of the helices correspond closely to those of the four EF-hands seen in the x-ray structure. Thus, myristoylated calcium-free recoverin and unmyristoylated recoverin with one Ca^{2+} bound have similar secondary structure. Further NMR studies are in progress to elucidate the three-dimensional structure of the myristoylated protein.

PERMEATION THROUGH MEMBRANE CHANNELS

W-PM-D1

ION PERMEATION MODELS OF RETINAL CGMP-ACTIVATED CURRENTS CARRIED BY SODIUM AND CALCIUM: COMPARISON OF ONE- AND TWO-SITE MODELS. ((G.B. Wells* and J.C. Tanaka)) Departments of Pathology* and Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104

The cGMP-activated cation channel from retinal rod cells, which changes the membrane potential in response to the effects of light, may have at least two binding sites for cations, based on several lines of experimental evidence, including concentration-dependent measurements of bi-ionic reversal potentials. Macroscopic current-voltage relations (IV's) from photoreceptor excised inside-out patches activated with saturating cGMP were fitted by a nonlinear least squares algorithm using the computational software *Mathematica* in an attempt to demonstrate statistically better fittings by a two site model compared to a one site model. The models, based on Eyring rate theory, did not include surface charge effects or ion-ion interactions. Single channel IV's were estimated by relaxing the voltage-dependence of macroscopic IV's to allow for channel gating and by estimating the number of channels in each patch based on a 25pS unitary conductance. In patches containing only Na, with the bath concentration varying from 20mM to 600mM, the energies of the barriers are 3.0-3.2RT (0.93-0.95 fractional distance δ; cytoplasmic side at δ=1) and 2.9-3.1RT (0.30-0.33 δ); the well is 8.6-8.9RT (0.62-0.67 δ). The two site model is not statistically better than the one site model. IV's, including reversal potentials, with the divalent cation Ca added to the bath at 1mM and 10mM are fitted better by a one site model than by two distinct binding sites, with the well for Ca approximately 17.5RT and barriers ≤1.1RT in the one site model.
Supported by NIH grant EY06640.

W-PM-D2

pH EFFECTS ON THE SUBCONDUCTANCE STATES OF THE CYCLIC NUCLEOTIDE-GATED CHANNEL FROM OLFACTORY EPITHELIUM. ((Michael Root and Roderick MacKinnon)) Program in Biophysics and Department of Neurobiology, Harvard Medical School, Boston MA, 02115.

In symmetric NaCl solutions, single cyclic nucleotide-gated channels from olfactory epithelium conduct Na^+ current at three distinct levels. The probability of being in any one of these conductance levels is strongly dependent on the pH of the extracellular solution. The lowest subconductance state predominates at low pH (<7.0) while the highest conductance level prevails at high pH (>8.0); the probability of being in the middle conductance level is greatest at pH 7.6. In addition, the conductance states are connected sequentially: transitions from one extreme conductance almost always dwell for some measurable time at the middle conductance level before proceeding to the other extreme conductance state. The data fit nicely to a theory involving two independent titratable sites each with a pK_a of 7.65 and each having an equivalent effect on the ionic current. The titratability has been mapped to a Glu residue in the putative pore region of the channel. A channel with Asp substituted for Glu still shows subconductances which respond to pH changes. However, replacement of Glu by Gly removes all subconductances from the channel as measured through a pH range from 5.5 to 9.

W-PM-D3

FRACTIONAL CALCIUM CURRENTS THROUGH RECOMBINANT GLUTAMATE RECEPTOR CHANNELS.
((N.Burnashev, Z.Zhou, E.Neher and B.Sakmann)) MPI für medizinische Forschung, Heidelberg, D-69120 and MPI für biophysikalische Chemie, Göttingen, D-37077, Germany.

Measurements of glutamate activated currents in host cells expressing recombinant glutamate receptor (GluR) channels suggests that their Ca^{2+} permeability relative to Na^+ or Cs^+ varies over a wide range and depends on the subunit composition of the channel. To assess the fractional cation current carried by Ca^{2+} through different types of recombinant GluR channels, we used simultaneous fluorescence and whole-cell current measurements during brief application of glutamate to HEK 293 cells expressing GluR subunits of AMPA, KA or NMDA families and loaded with fura-2 (1mM). To calibrate the Ca^{2+}-sensitive fluorescence signal as a measure of the pure Ca^{2+}-influx charge, HEK 293 cells expressing Ca^{2+} permeable GluR-A homomeric channels were bathed in a solution containing 10 mM Ca^{2+} and the impermeant cation N-methyl-D-glucamine as the major cation on both the outside and the inside of the cell. The results showed that the amount of the fractional Ca^{2+} current mediated by GluR channels in physiological Ca^{2+} concentration (1.8 mM) varied between 0.5% and 12%, depending on the GluR channel subunit family, the subunit combination and membrane voltage.

W-PM-D4

CYSTEINE SUBSTITUTION AND COVALENT MODIFICATION OF AMINO ACIDS IN THE PORE OF A CYCLIC NUCLEOTIDE-GATED CHANNEL.
((Zhong-ping Sun, Evan H. Goulding, Myles Akabas, Arthur Karlin, and Steve A. Siegelbaum)) Depts. Pharmacol., Physiol., Ctr. Neuro. & Behav., & Ctr. Molec. Recog., HHMI, Columbia University, New York, NY 10032

Cyclic nucleotide-gated (CNG) channels share sequence and structural features with voltage-gated channels, all of which contain six putative transmembrane domains (S1 to S6) and the H5 domain (also called P segment). H5 contributes to the lining of the pore. We combined site-directed mutagenesis and covalent modification to investigate the proposed structure of the H5 domain in the cloned bovine retinal CNG channel (RET). The positively charged methanethiosulfonate derivative MTSEA ($CH_3SO_2SCH_2CH_2NH_3^+$) specifically modifies cysteine residues by adding the -$SCH_2CH_2NH_3^+$ moiety to free -SH groups to form mixed disulfides. We mutated, one at a time, consecutive residues in the H5 domain of RET to cysteine and expressed the mutants in *Xenopus* oocytes. We assume only those cysteines lining the pore will be accessible and, if MTSEA reacts with a cysteine in the pore, ion conduction will be irreversibly altered. From inside-out and outside-out patch-clamp recordings, we have identified a specific threonine residue which contributes to the pore and is accessible from the extracellular side and another threonine residue accessible only from the intracellular side. These results are consistent with the view that the H5 domain forms a loop that dips into the membrane. (Supported by NIH, MDA, AHA, and Klingenstein Foundation)

W-PM-D5

THE VOLTAGE GATING OF VDAC IS SENSITIVE TO ION FLOW.
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Unlike transistors, voltage-sensitive channels are stricly voltage-dependent devices. This may not be correct for mesoscopic channels. VDAC is a voltage-gated channel from the mitochondrial outer membrane. It has two gating processes: one at positive and the other at negative potentials. The energetics of VDAC gating are quite different when measured in the presence or absence of an ion gradient. A positive potential on the high-salt side, results in channel closure at lower transmembrane potentials. The mid-point potential (Vo) shifted from 25 to 4.8 mV with an activity gradient for KCl of 1 vs 0.1 molal. The opposite occurred for negative potentials on the high-salt side (Vo shifted from -25 to -28.5 mV). Thus the salt gradient favored closure for one gating process and opening for the other. These results could be explained if part of the electrochemical potential of the gradients present were transferred to the gating mechanism. If the kinetic energy of the ion flow were coupled to the gating process, the effects of the gradient would depend on the mass and velocities of these ions. This was tested using a series of different salts (KCl, NaCl, LiCl, KBr, KAcetate, NaButyrate and RbBr) under an identical activity gradient (1:0.1 molal). The kinetic energy correlated very well to the measured shifts in free energy of the channel gating. This was true for both polarities. Thus the gating of VDAC is influenced by ion flow. These results are consistent in sign and direction with the voltage gating process in VDAC which is believed to involve the movement of a positively-charged portion of the wall of the channel out of the membrane. (Supported by ONR grant # N00014-90-J-1024)

W-PM-D6

SELECTIVE ION BINDING IN SODIUM AND CALCIUM CHANNELS
((Rachel Brem, Steve Bogusz, and David Busath)) BROWN UNIVERSITY, BOX G-B3, Providence, RI 02912 (Spon. by K. Chapman).

Mutations of the highly conserved E's from repeats I-IV of the Ca^{++} channel and the homologous D,E,K,&A from the Na^+ channel suggest that these residues may cooperate in selectively coordinating transported ions through the dehydrated stage of passage. Using empirical force field, atomistic modeling of a β-barrel structure, like that proposed for the potassium channel (Bogusz and Busath, 1992), with these four residues projecting into the barrel from a plane perpendicular to the barrel axis, we have analyzed the expected binding selectivity and protonation for these two types of binding sites and find them consistent with expectations. At least one of the Ca^{++}-site glutamates would probably be protonated in the absence, but not in the presence of an ion in the site. A model cyclic dodecapeptide with backbone diameter similar to the walls of the β-barrel and four inward pointing anionic E's (spaced by P-P dipeptides) was analyzed by conformational search for ideal backbone and Ca^{++}-binding configurations. The backbone energy was not increased upon cyclization, suggesting that the synthesis should be feasible. Neglecting the water interactions, the lowest potential energy structures had circular backbone, Ca^{++} at the center, and Ca^{++} coordination by the four E's. In 15 A water droplets, the results were ambiguous with similar potential energies for Ca^{++} coordination by combinations of 0 to 4 E's and multiple backbone carbonyls. The results suggest that the coordination of Ca^{++} by a small peptide is a delicate balance of solvation, ion-attraction, and side-chain mutual repulsion factors. Nevertheless, the proposed dodecapeptide mimic of the Ca^{++} channel binding site (and the Na^+ site mimic analog) could be a useful model for ion binding in the channels.

W-PM-D7

A CONDUCTANCE MAXIMUM OBSERVED IN AN INWARD-RECTIFIER POTASSIUM CHANNEL (ROMK1).
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One prediction of a multi-ion pore is that it should show a maximal conductance value as the permeant ion concentration is varied equally on both sides of the membrane. Such maxima have been observed in gramicidin and in a Ca^{2+}-activated K^+ channel at extremely high ion concentrations (> 1 M). In the present study we examined the conductance-activity relationship in an inward-rectifier K^+ channel, ROMK1. Single channels, expressed in Xenopus oocytes, were studied using inside-out patch recording in the absence of internal Mg^{2+} to eliminate blockade of outward current. The K^+ activity on both sides of the membrane was varied over a range of 10 to 600 mM. As K^+ was raised from 10 mM, the conductance increased steeply and reached a maximum value (39 pS) at 200 mM. The single channel conductance then became progressively smaller as K^+ was raised beyond 200 mM. At 600 mM K^+, the conductance was reduced to 77% of its maximum value. The shape of the conductance-activity curve observed in the ROMK1 channel implies that it has multiple K^+-occupied binding sites in its conduction pathway.

W-PM-D8

SELECTIVITY DIFFERENCES BETWEEN IRK1 AND ROMK1, TWO INWARDLY RECTIFYING K^+ CHANNELS.
((M. Taglialatela, B. Wible, R. Caporaso and A.M. Brown)) Dept. Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

Inwardly rectifying K^+ channels maintain the resting potential close to E_k and help terminate prolonged depolarizations. Recently, molecular cloning of the cDNA's encoding three inwardly rectifying K^+ channels ROMK1 (Ho et al., Nature 362: 31-38, 1993), IRK1 (Kubo et al., Nature 362: 127-133, 1993) and GIRK1 (Kubo et al., Nature 364: 802-806, 1993) has been reported. The predicted amino acid sequences from these genes reveal the presence of two transmembrane domain with an intervening sequence which is similar to the H5 region of voltage-gated outwardly-rectifying K^+ channels. In order to gain insight into the molecular mechanism of ion permeation of inwardly rectifying K^+ channels, we investigated the selectivity properties of IRK1 and ROMK1. These channels were expressed in *Xenopus* oocytes and reversal potentials were measured with two microelectrode voltage-clamp under bi-ionic conditions with 100 mM K^+, Rb^+, NH_4^+, Cs^+, Na^+ or Li^+ in the bathing solution. The following permeability ratios, calculated from E_x-E_k=2.303 RT/F log P_x/P_k, were obtained:

P_x/P_k	IRK1	ROMK1
PNH_4^+/PK^+	4.27±0.44	0.33±0.06
PRb^+/PK^+	0.13±0.05	0.71±0.10
PCs^+/PK^+	0.02±0.01	0.25±0.06
PNa^+/PK^+	0.01±0.006	0.045±0.01
PLi^+/PK^+	0.008±0.004	0.039±0.08

We are currently investigating the molecular basis for these permeability differences, particularly the high NH_4^+ permeability in IRK1.
(Supported by HL 36930 and TXATP 0032 grants to AMB).

W-PM-D9

MODELS OF THE OUTER VESTIBULES AND SELECTIVITY FILTERS OF Na^+, Ca^{2+}, AND K^+ CHANNELS. ((H. Robert Guy and Stewart R. Durell)) Laboratory of Mathematical Biology, DCBDC, NCI, NIH, Bethesda, MD. 20892.

In our latest three-dimensional models of Na^+, Ca^{2+}, and K^+ channels, the first part (SS1) of the P segment and a segment near the end of P are α helices. These helices form most of a cone-shaped outer vestibule. The helices are amphipathic in Na^+ and Ca^{+2} channels and their hydrophilic residues line the vestibule. The ion selective region is formed by residues linking the helices; in *Shaker* K^+ channels the linking sequence is TTVGYGD and in Ca^{2+} channels it is TXEXW in all four repeats. The linking segments contain β turn and random coil type conformations in all models. In some models of K^+ channels, the backbone amide oxygens of the GYG residues form a series of ion binding sites along the pore's axis. The narrowest part of the Ca^{2+} pore is formed by the four (one/repeat) glutamate carboxyl groups; in Na^+ channels the four 'selectivity' residues are Asp, Glu, Lys, and Ala from repeats I-IV respectively. Tetrodotoxin and saxitoxin fit tightly into the portion of outer vestibule formed by the connecting segments; their guanidium group interacts with the 'selectivity' residues and most of their other atoms interact favorably with the three residues in each repeat that follow the 'selectivity' residues. The ion selective regions are comprised of residues that are conserved among distantly related channels with the same selectivity. The models are consistent with numerous experimental results.

STIMULUS-SECRETION COUPLING I

W-PM-E1

Correlation between the kinetics of $Fc_\epsilon RI$ dimerisation by monoclonal antibodies and secretory response in mast-cells. ((R. Schweitzer-Stenner[1], E. Ortega[2], I. Pecht[2])) [1]Inst. of Exp. Physics, Univ. of Bremen, 28359 Bremen, Germany and [2]Dep. of Chem. Immunology, The Weizmann Institute of Science, Rehovot 76100. (Spon. by H. Eisenberg)

Rates of association and dissociation of monoclonal, IgG class antibodies (mAbs) (F4, J17 and H10) to and from the type 1 Fc_ϵ receptor on RBL-2H3 mast cells ($Fc_\epsilon RI$) were measured. These mAbs dimerize the $Fc_\epsilon RI$. The data were best fitted by a two-steps model involving a conformational transition from a low (l) to a high (h) affinity state of the receptor-ligand complex (*Ortega* et al., Biochemistry, *30*, 3473, **1991**). Thus the following $Fc_\epsilon RI$-dimer spe-cies are considered: all l- and h-state dimers, D_{ll} and D_{hh} and a hybrid D_{lh} with one receptor in the l and the other in the h-state. Different dimer formation rate constants were derived for each of the three mAbs. This was interpreted in terms of $Fc_\epsilon RI$-mAb complexes being subjected to different orientational constraints. By utilizing a theoretical model proposed by *Dembo* et al. (J. Immunol. *128*, 1864, **1979**) the time course of $Fc_\epsilon RI$ dimerization and the rates of cellular secretory response to the three employed mAbs were compared. $Fc_\epsilon RI$ dimers were found to require life-times longer than a threshold value in order to be effective secretagou-ges. This condition is met by D_{lh} and D_{hh}, whereas D_{ll} decays too fast. Moreover desensitization might take place at high receptor dimer concentrations. As a consequence H10 and J17 become less effective in triggering secretion than F4 in spite of their better dimerizing capacity.

W-PM-E2

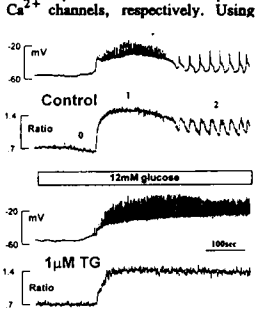
TRH AND PROTEIN KINASE C MODULATION OF VOLTAGE DEPENDENT SECRETION AND CALCIUM CHANNEL CURRENTS IN RAT LACTOTROPHS. ((A.F. Fomina and E.S. Levitan)) Dept. of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261.

Stimulation of prolactin secretion by TRH (thyrotropin-releasing hormone) is associated with activation of protein kinase C (pkC). However, the role of voltage-gated Ca^{2+} channels in the control of prolactin secretion is not clear. In this study we used the perforated patch method and time domain membrane capacitance measurements to assay exocytotic secretion from single immunologically identified rat lactotrophs. Bath application of 1 μM TRH stimulated secretion at -70 mV and/or increased secretion evoked by 0.5-1 s depolarizations to +10 mV. 100-500 nM Phorbol 12-myristate 13-acetate (TPA, a pkC activator), but not by 4-α-TPA, also enhanced depolarization evoked secretion. Depolarization evoked secretion was always blocked by bath application of Co^{2+} and was not seen when the membrane potential was stepped to +50 mV, indicating participation of voltage-gated Ca^{2+} channels. Ba^{2+} currents were measured to test if Ca^{2+} channels are modulated. TRH induced a biphasic effect on high voltage activated (HVA) currents: within seconds the current decreased by 15-40%, followed by a slow recovery (2-4 min) to a level 5-20% higher than before application of the transmitter. TPA increased HVA current amplitude by 20-100% during 2-4 min applications. Low voltage activated currents were not affected by TRH or TPA. These findings suggest that (i) pkC may be involved in enhancement of depolarization evoked secretion by TRH and (ii) modulation of HVA Ca^{2+} channels might contribute to the sustained phase of TRH-induced prolactin secretion. Supported by the NIH, Klingenstein Foundation and AHA (PA affiliate).

W-PM-E3

INTRACELLULAR CALCIUM STORE REGULATION OF MEMBRANE POTENTIAL IN MOUSE ISLETS; EFFECTS OF THAPSIGARGIN. ((I.D.Dukes, M.S. McIntyre, R.J. Mertz, M.W. Roe[1] and J.F. Worley III)) Glaxo Research Institute, RTP, NC 27709 and [1]University of Chicago, Chicago IL 60637. (Spon. C. Foster)

Glucose stimulation of pancreatic islets is associated with a triphasic change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (0, 1 and 2) brought about by stimulation of $Ca^{2+}{}_i$ sequestration, $Ca^{2+}{}_i$ release and phasic influx through voltage activated Ca^{2+} channels, respectively. Using simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential in single mouse islets loaded with fura-2, we demonstrate that $Ca^{2+}{}_i$ store release by glucose, causes a depolarization which acts to maintain Ca^{2+} influx through Ca^{2+} channels. Prevention of store refilling with thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, maximized this signal leading to sustained depolarization and elevated $[Ca^{2+}]_i$, implying that store depletion was responsible for the depolarizing signal. The sustained $[Ca^{2+}]_i$ rise induced by glucose following thapsigargin treatment was voltage dependent, as indicated by the suppressive effects of diazoxide. Exposure to EGTA ablated the $[Ca^{2+}]_i$ rise but not the depolarization, demonstrating that the store depletion-activated depolarizing current was not the source of the maintained $[Ca^{2+}]_i$. Thus, the depolarizing signal acts to keep the membrane depolarized in the optimum voltage range for Ca^{2+} influx through voltage gated Ca^{2+} channels, and is likely to be a physiological mechanism for repleting $Ca^{2+}{}_i$ stores and regulating insulin secretion.



W-PM-E4

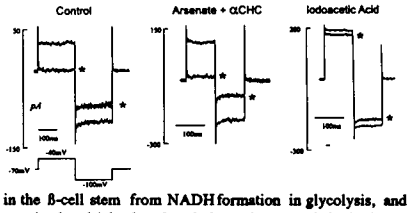
INTRACELLULAR CALCIUM STORE DEPLETION ACTIVATES A MEMBRANE CURRENT IN MOUSE β-CELLS. ((J.F. Worley III, M.S. McIntyre, B. Spencer & I.D. Dukes)) Glaxo Res. Institute, RTP, NC 27709.

$[Ca^{2+}]_i$ oscillations in many non-excitable cells are produced by release of calcium from intracellular stores which replenish by signaling the plasma membrane to permit calcium influx. Islets of Langerhans also show $[Ca^{2+}]_i$ oscillations when exposed to stimulatory levels of glucose which cause insulin secretion. In this study, we have examined the influence intracellular stores on glucose-induced membrane potential oscillations and membrane currents in mouse islets and β-cells. In islets, intracellular store modulation by caffeine, thapsigargin and varied extracellular Ca^{2+} levels, altered membrane potential oscillatory patterns in a glucose-dependent manner. Caffeine (5mM) slowed the oscillations while inducing membrane hyperpolarization and lowering $[Ca^{2+}]_i$. Exposure of islets to 50μM extracellular calcium caused a glucose-dependent train of reduced amplitude action potentials superimposed on a maintained depolarization, while $[Ca^{2+}]_i$ increased. Maitotoxin (1-10pg/ml, a stimulator of IP_3 production) produced a glucose-independent rise in $[Ca^{2+}]_i$. These data correlate depletion of intracellular calcium stores with membrane depolarization and store sequestration with hyperpolarization. In support of these observations, an inwardly rectifying cationic current was present in single β-cells upon exposure to thapsigarin, low external calcium and maitotoxin. The properties of this current were distinct from voltage-dependent calcium currents but similar to the store depletion-activated currents described in non-excitable cells. These data are consistent with depletion of intracellular stores producing membrane depolarization and sequestration events enhancing depolarization through modulation of a glucose-sensitive cationic current in β-cells.

W-PM-E5

NADH PRODUCED DURING GLYCOLYSIS MEDIATES GLUCOSE-DEPENDENT CHANGES IN VOLTAGE AND Ca^{2+} IN MOUSE ß-CELLS AND ISLETS. ((I.D.Dukes, B. Spencer, M.W. Roe[1], R.J. Mertz, M.S. McIntyre, L.H.Philipson[1] &J.F. Worley III.)) Glaxo Res. Institute, RTP NC 27709 and University of Chicago, Chicago IL 60637.

Increases in cytosolic ATP following glucose metabolism have been considered the key metabolic signal in initiating insulin secretion, by inducing closure of ATP-dependent K^+ channels (K_{ATP}), thereby leading to phasic membrane depolarizations and an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$).We report here that the critical signalling step by which glucose initiates changes in ß-cell K_{ATP} channel activity, membrane potential and $[Ca^{2+}]_i$ are mediated neither by ATP produced directly during glycolysis nor by the operation of the Krebs cycle. Thus, glucose-induced block of K_{ATP} currents (*) was unaffected by either arsenate or by the pyruvate transport inhibitor, 2α-methoxy-cyanocinammate (α CHC). Instead, the signal appears to stem from a single step in glycolysis with the production of NADH during the oxidation of glyceraldehyde-3-phosphate. Inhibitors of NADH formation (iodoacetic acid) or NADH - dependent mitochondrial ATP production both suppressed glucose-induced alterations in membrane currents in ß-cells, and depolarization and $Ca^{2+}{}_i$ transients in islets. In conclusion, early glucose signalling steps in the ß-cell stem from NADH formation in glycolysis, and its processing to form ATP at mitochondrial phosphorylation sites 1 and 2 via the operation of discrete shuttles.



W-PM-E6

DOES OMEGA CONOTOXIN MVIIA BLOCK INSULIN SECRETION BY BLOCKING L-CHANNELS ?

((L. S. SATIN, J. TEAGUE* AND S.J. TAVALIN)). Dept. of Pharm./Tox., Med. Coll. of VA., Richmond,VA. 23298 and *Univ. of Wash., Seattle, WA. 98115.

To understand the role of different Ca channels in the control of insulin secretion, we tested the action of omega conotoxin MVIIA on Ca current and glucose-dependent insulin secretion using HIT cells. Ca current was measured using standard whole-cell patch clamp techniques, while insulin secretion was measured using a static incubation protocol. Cells were preincubated for 2 hours in 0 glucose Krebs-Ringer bicarbonate (KRB) followed by 1 hour in KRB containing test incubates. Released insulin was determined by collecting the KRB that bathed the cells, while cellular insulin content was obtained from acid-extracts of the cell layer. Secretion was then expressed as % fractional release. Samples were assayed against human insulin standards using conventional RIA. Application of 1 µM MVIIA reversibly blocked 56.3 ± 10.3% of the Ca current at +10 mV (X±S.E.; N=5). MVIIA over the concentration range of 0.1 to 10 µM inhibited glucose-dependent secretion by 16.8 to 66.0% (in 16.6 mM glucose), with a K_i of ≈5 µM. If this inhibition was due to blockade of N-type Ca channels, a component of secretion should be insensitive to L-channel blockers. Instead, glucose-dependent secretion was dose-dependently inhibited by nimodipine (K_i ≈3.5 nM), with total block at 0.1 µM. The L-channel agonist BAY K 8644 potentiated secretion and reversed the inhibition produced by 1 µM nimodipine. In summary, it appears likely that Ca influx into insulin-secreting HIT cells is mediated by a conotoxin- and dihydropyridine-modulated L-type Ca channel.

W-PM-E7

PHASE-RESETTING OF THE GnRH-INDUCED OSCILLATIONS OF CALCIUM-DEPENDENT POTASSIUM CURRENTS BY SINGLE DEPOLARIZING PULSES IN RAT PITUITARY GONADOTROPHS ((L. Vergara, S.S. Stojilkovic and E. Rojas)) LCBG,NIDDK and ERRB,NICHD,NIH, Bethesda, MD 20892. (Spon. by I. Atwater)

The neuropeptide GnRH elicits cytosolic $[Ca^{2+}]_i$-oscillations in cultured rat pituitary gonadotrophs. The mechanism of these oscillations depends on $InsP_3$-mediated Ca^{2+} release from intracellular stores, but the maintenance of the response requires the influx of calcium through voltage-sensitive Ca^{2+}-channels (VSCC). The objective of the present work was to study the effect of single brief transients of calcium influx on the timing of the oscillatory cycle. The nystatin-perforated whole cell recording configuration of the patch-clamp technique was used to record the $[Ca^{2+}]$-dependent, apamin sensitive, potassium current ($I_{K(Ca)}$) as an indicator of the $[Ca^{2+}]_i$. Before the application of GnRH, a brief (100 ms) depolarizing pulse (50 mV, from a holding potential of -50 mV) generated a small (10-20 pA) outward $K_{(Ca)}$-tail current. In the presence of the agonist (10^{-10} - 10^{-9} M) the cells responded to a single brief depolarization in a biphasic manner. During the initial phase, this pulse had no effect on the timing of the GnRH-induced oscillations. However, during the sustained phase, the stimulus induced a $K_{(Ca)}$ current transient (50-100 pA), similar to the agonist-evoked oscillations both in amplitude and shape. Decreasing the time lapse between the pulse and the preceding oscillation induced a negative phase shift of the successive oscillations, without any significant change in the endogenous frequency. The initial lack of response to a single depolarizing pulse observed in the presence of GnRH may be related to the amount of calcium in the intracellular-stores. We concluded that during the last phase of the response to GnRH, brief transients of Ca^{2+} influx can reset the phase of the oscillations without affecting the frequency of the cytosolic oscillator. This suggests that Ca^{2+}-release can be induced by Ca^{2+}-dependent activation of the IP_3-receptor channel, but the period of the oscillations is determined by an endogenous mechanism.

W-PM-E8

EXPRESSION OF THE HUMAN K+ CHANNEL HKV1.5/HPCN1 IN PANCREATIC BETA CELLS OF TRANSGENIC MICE IS ASSOCIATED WITH HYPERGLYCEMIA. ((L.H. Philipson, A. Kusnetsov, I. Westerman, J.F. Worley III, I.D. Dukes[1], and M. Rosenberg[1],)) Univ. of Chicago, Chicago, IL 60637 and [1] Glaxo Res. Inst., R.T.P., N.C., 27709

We have previously described cDNA and genomic clones encoding a human *Shaker*-like K+ channel, termed hPCN1/Kv1.5, found to be over-expressed in a human insulinoma and also reported in heart, brain, and pituitary-derived GH3 cells. To characterize the effect of overexpression of this channel in normal ß-cells, a transgene was constructed in which the hPCN1/Kv1.5-c-peptide fusion gene is driven by the rat insulin promoter II. This construct was initially employed in transient transfection experiments with BTC3 mouse insulinoma cells. Recordings from positive cells by whole cell voltage clamp revealed K+ currents of at least two fold greater amplitude than background outward K+ currents, with faster activation kinetics, sensitive to clofilium, all consistent with the expression of hPCN1/Kv1.5. The linearized transgene was then injected into the male pronucleus of fertilized oocytes. Southern blots of tail DNA were positive in 7 of 123 potential founders. Of these, three males (Nos. 1651, 1663, 1669) were found to be hyperglycemic when tested at three months of age. All seven founders were then bred with wild type C57BL/6J to create multiple F1 lines. Only animals descended from the three male founders continued to show hyperglycemia. Blood sugars for the positive animals have ranged as high as 350-450 mg/dl. Fasting sugars, however, of tg+ and negative animals have been lower, in 70-100 mg/dl range. Western blots of islets from tg+ and control mice probed with anti-human c-peptide antisera confirmed the expression of the hPCN1-c-peptide fusion protein in only tg+ islets. This transgenic model may prove to be a useful system in which to analyze the functional consequences of a pharmacologically distinct delayed rectifier K+ channel on the function of an excitable cell, the pancreatic ß-cell.

W-PM-E9

INHIBITING THE RUNDOWN OF Cl AND K CHANNELS IN EXCISED PATCHES. ((F. Becq[1], T.J. Jensen[2], J.A. Tabcharani[1], J.R. Riordan[2] and J.W. Hanrahan[1]))
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Many ion channels deactivate spontaneously in excised patches due to dephosphorylation by membrane-associated phosphatases. We have used this rundown to assay the activity of phosphatases regulating the CFTR Cl channel (*Nature* 352:628;1991) and the carbachol-stimulated K channel in secretory epithelia (*FASEB J.* 7:A351,1993). The mean duration of CFTR activity after excision from forskolin-stimulated cells was 77 seconds (22°C). Rundown was slowed slightly by ATP and reversed by addition of PKA. The time required for rundown was not affected by okadaic acid or calyculin A but was increased 4-fold by vanadate, an inhibitor of phosphoryl transfer reactions, and by Br-tetramisole (Br-t), a potent alkaline phosphatase inhibitor. The widely-used phosphodiesterase antagonists IBMX and theophylline, which reportedly inhibit phosphatases in some systems, were also potent inhibitors of rundown (*FEBS Lett.* 257:337;1993). By contrast, phosphodiesterase antagonists that do not inhibit phosphatases in other systems such as caffeine and dipyridamole had no effect on rundown. Immuno-precipitated CFTR was dephosphorylated *in vitro* by exogenous alkaline phosphatase. Dephosphorylation of CFTR in isolated membranes was inhibited by IBMX or Br-t, suggesting the channel itself is a substrate for the endogenous phosphatases that cause rundown. Similar phosphatases apparently regulate the inwardly rectifying K channel at the basolateral membrane; its rundown was also inhibited by IBMX but not by okadaic acid. We conclude: 1) phosphatase regulation of these channels is dynamic, 2) the endogenous phosphatase activity resembles alkaline phosphatases more than other known protein phosphatases, 3) activation of mutant CFTR channels by high concentrations of IBMX may be due, in part, to phosphatase inhibition, 4) IBMX and Br-t may be useful in diverse studies of phosphorylation/regulation of membrane proteins.
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