Tu-Pos98

IONIC CONDUCTANCES OF IDENTIFIED RAT SOMATOTROPH CELLS STUDIED BY PERFORATED PATCH RECORDING ARE MODULATED BY GROWTH HORMONE SECRETOGOGUES. R. J. Leonard, L. -Y. Chaung, and S. -S. Pong. Dept. of Membrane Biochemistry & Biophysics and Dept. of Growth Biochemistry & Physiology, Merck Sharp & Dohme Research Labs, Rahway, NJ 07065.

We have used the "perforated patch" recording technique of Horn & Marty (JGP 92:145-159, 1988) to examine the modulation of ionic conductances by a growth hormone secretogogue in identified rat somatotroph cells *in vitro*. Primary cultures of rat pituitary cells were subjected to a reverse hemolytic plaque assay procedure to positively identify growth hormone (GH) secreting cells. Following this procedure, somatotroph cells appear as isolated 'islands' in a sea of erythrocytes. Whole-cell voltage and current records were obtained from such isolated, identified cells using the nystatin-permeablized patch technique, rather than conventional whole-cell voltage clamp, to prevent loss of intracellular constituents from internal perfusion. Voltage clamp records revealed voltage dependent Na^+ currents that were blocked by TTX; voltage dependent Ca^{++} currents; Ca^{++}-activated, charybdotoxin-sensitive K^+ currents, and TEA-sensitive, voltage dependent K^+ currents. Under passive voltage (zero-current) recording, some cells exhibited spontaneous action potentials, with most cells displaying slow oscillations in membrane potential around -40 mV. Application of the synthetic hexapeptide GH secretogogue 'GHRP-6' caused depolarization and an increase in spike frequency. Under voltage clamp, GHRP-6 appeared to inhibit a voltage-dependent outward current. Electrophysiological effects of growth hormone releasing factor (GRF) and somatostatin (SRIF) were also observed.

Tu-Pos99

CYCLIC GMP-DEPENDENT PROTEIN KINASE REGULATION OF CHLORIDE CHANNELS IN T_{84} CELLS

Meiqiu Lin[1], Angus C. Nairn[2] and Sandra E. Guggino[1,3]
Department of Medicine[1] and Neuroscience[3], The Johns Hopkins University School of Medicine, Baltimore, MD 21205. Laboratory of Molecular and Cellular Neuroscience[2], The Rockefeller University, New York, NY 10021-6399

The chloride-secreting human colonic cell line, T_{84}, exhibits chloride short circuit current stimulated by heat stable toxin from E. coli (STa). STa increases cyclic GMP by hydrolysis of GTP. In other chloride secreting epithelia, like tracheal airway cells, a 35 pS rectifying chloride channel is opened by cyclic AMP-dependent protein kinase (PKA) or protein kinase C. Until now activation of chloride channels by cyclic GMP-dependent mechanisms has not been explored. Using the single channel patch clamp technique, patches were excised into a low Ca^{2+} (<50 nM) Ringer solution. When 200 nM cyclic GMP-dependent protein kinase (PKG) isolated from smooth muscle, 100 μM cGMP, and 1 mM ATP were added to the bath (intracellular side), chloride channels opened within 1-10 minutes. The same experiment was also performed without PKG (100 μM cGMP and 1 mM ATP). All experiments were repeated with 2-10 μM Walsh inhibitor. Voltage depolarization (n=4) opened 35 pS outwardly-rectifying chloride channels. However, cGMP and ATP with (n=5) or without exogenous PKG (n=8) activated channels with a single channel conductance of 12 pS, a linear I-V relationship, a low open probability (Po), and a short mean open time with rapid fluctuations between open and closed states. When the membrane potential was depolarized to a positive voltage at the end of PKG experiments, both 12 pS and 35 pS channels were present. Conversely, if voltage depolarization was followed by cGMP and ATP, the 12 pS channel appeared after 8 minutes (n=2). This indicates that a linear chloride channel is activated by either endogenous or exogenous PKG.

Tu-Pos100

IONIC CONDUCTANCES ACTIVATED DURING VOLUME REGULATION IN MDCK CELLS: A PATCH-CLAMP STUDY. U. Banderali and G. Roy. Groupe de Recherche en Transport Membranaire. Université de Montréal. Montréal, Qué. H3C 3J7.

Patch-clamp studies have been performed on MDCK epithelial cultured cells in order to characterize the ionic channels participating in the process of volume recovery (RVD), after the exposure of the cells to a hypotonic medium. Subconfluent cultures have been employed. The results of experiments carried out in the on-cell configuration with a 135 mM KCl pipette solution show that up to four different ionic channels are activated after the hypotonic shock. Some of these channels are likely to be K^+ channels. The results of parallel experiments carried out in the on-cell and in the inside-out configurations allowed a rather detailed description of a particular K^+ channel. Its conductance has been evaluated to be 27.4±3.3 pS, its selectivity ratio P_{Na}/P_K is .042, and its activity is not blocked by 1 mM quinine. An increase in the open probability (P_O) is observed almost immediately after the hypotonic shock in this K^+ channel. Similar experiments carried out with a 150 mM Choline-Chloride pipette solution, revealed a Cl^- selective channel also participating in the RVD. This channel has a conductance of 52.7±7.45 pS and undergoes an increase in P_O both after the hypotonic shock and after the subsequent return to the isotonic medium. A delay of approximately 1.30 min is observed when this increase is induced by the hypotonic shock. All these data are in good agreement with the results of a previous work whose purpose was to study the changes in membrane potential and in ionic contents during the process of volume regulation in MDCK cells (Roy & Sauvé, J. Memb. Biol. 100, 83-96, 1987).

Tu-Pos101

CYCLIC GMP AND SODIUM NITROPRUSSIDE INDUCE A NON-SELECTIVE CATION CHANNEL IN A RENAL EPITHELIAL CELL LINE (A6).

Yoshinori Marunaka*, Akito Ohara, Paul Matsumoto and Douglas C. Eaton. Dept. of Physiol., Emory Univ. Sch. Med., Atlanta, Georgia 30322, USA. *Div. of Respiratory Research, The Hospital for Sick Children Research Institute, Toronto, Ontario M5G 1X8, Canada.

The effects of cyclic GMP (cGMP) and sodium nitroprusside (SNP) on ion channels in a renal distal nephron cell line (A6) were investigated using single channel recording techniques. Confluent A6 cells used for single channel recording were cultured on permeable collagen films for 10 ~ 14 days. All patches were formed on the apical membrane of A6 cells. In some cell-attached patches, there were no channel activity; i.e., no ionic current was detected. In these cases, we applied SNP to a final concentration of 30 μM. About ten to fifteen minutes after adding SNP to the luminal solution (outside the cell-attached patch) some channel activity was observed in the patch membrane, which originally had no detectable channel activity. The single channel conductance of the SNP-induced channel was about 1 pS and the ion permeability of the channel was similar for Na and K and very low for Cl compared to Na or K. It is thought that SNP increases cellular cGMP level, so we applied cGMP to inside-out patches. Several minutes after adding cGMP to the cytosolic face of the patch membrane which originally had no channel activity, we detected single channel currents whose single channel conductance and ion selectivity were very similar to the SNP-induced channel. These observations suggest that SNP induces a non-selective cation channel with small single channel conductance of 1 pS via an increase in cGMP which directly induces or activates the channel.

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Tu-Pos102

ABSCISIC ACID ACTIVATION OF NON-SELECTIVE Ca^{2+} PERMEABLE CHANNELS CONTRIBUTES TO REPETITIVE Ca^{2+} TRANSIENTS IN GUARD CELLS. Julian I. Schroeder, Dept. of Biology, University of California, San Diego, La Jolla, CA 92093.

The growth regulator abscisic acid closes stomatal pores. Indirect evidence suggests that Ca^{2+} flux into the cytoplasm of guard cells is a major factor in the induction of stomatal closing. To directly investigate initial events in abscisic acid-induced signal transduction in guard cells, simultaneous fluorometric measurements of cytosolic Ca^{2+} using fura-2 and whole-cell patch clamp recordings were performed on *Vicia faba* guard cells. Resting cytosolic Ca^{2+} concentrations were in the range of 0.2 μM. In responsive guard cells, exposure to external abscisic acid produced transient repetitive increases in the cytosolic free Ca^{2+} concentration. Ca^{2+} transients were accompanied by concomitantly occurring increases in an inward-directed ion current. Depolarization of the membrane terminated repetitive elevations in the cytosolic free Ca^{2+} concentration and reversed transient currents, which were activated by abscisic acid. These findings suggest that abscisic acid-mediated Ca^{2+} transients were produced by influx of Ca^{2+} via Ca^{2+}-permeable channels. Reversal potentials of abscisic acid-induced currents demonstrate that these currents are not highly Ca^{2+}-selective allowing the permeation of both Ca^{2+} and K^+. The repetitive activation pattern of abscisic acid-activated ion channels during continuous abscisic acid application suggests that these ion channels may require intermediate coupling mechanisms. Abscisic acid-activated Ca^{2+}-permeable channels produce elevations in the cytosolic Ca^{2+} concentration, which in turn can modulate cellular mechanisms promoting stomatal closure.

Tu-Pos103

AMINO ACID - AND SECOND MESSENGER-INDUCED RESPONSES IN ISOLATED CATFISH OLFACTORY NEURONS. Takenori Miyamoto, Diego Restrepo and John H. Teeter, Monell Chemical Senses Center, and Univ. of PA, Philadelphia, PA 19104.

An odorant-induced adenosine cyclic monophosphate (c-AMP) cascade has been implicated in olfactory transduction. Recently, studies in the channel catfish (Ictalurus punctatus) have shown that exposure of isolated olfactory cilia to olfactory stimuli (L-amino acids) leads to increases not only in c-AMP but also in inositol-1,4,5-trisphosphate (IP_3) in the cilia. Correspondingly, both c-AMP- and IP_3-gated channels were observed in isolated ciliary membranes incorporated into phospholipid bilayers. In addition, L-amino acids elicit a transient elevation in intracellular calcium in a subset of olfactory neurons. We recorded the responses of isolated catfish olfactory neurons to L-amino acids and second messengers using the patch-clamp technique to characterize the transduction pathways underlying the olfactory response. The responses to L-amino acids were categorized into three different types by their voltage dependence and sensitivity to drugs: an inward-rectifying Ca^{2+} - and ruthenium red-sensitive response (Type I), an amiloride sensitive response (Type II) and an outward-rectifying response which is insensitive to both amiloride and ruthenium red (Type III). On the other hand, both 10 μM IP_3 and c-AMP included in the patch pipette induced transient depolarizing responses. The responses became sustained when buffering capacity for Ca^{2+} in the pipette solution was increased. Sensitivity of these responses to some drugs suggests that the Type I and III responses correspond to IP_3- and c-AMP-induced responses, respectively. Our results support the hypothesis that there are multiple transduction pathways in the catfish olfactory system.

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Tu-Pos104

MEMBRANE POTENTIAL AND INTRACELLULAR CALCIUM AS REGULATORS OF L-TYPE CALCIUM CHANNEL NUMBER. Alfredo R. Narvaez and Richard McGee, Jr. Dept. of Pharmacol., Medical College of Ohio, Toledo, Ohio 43699.

The role of membrane potential in regulating L-type Ca^{2+} channel number in the aortic cell line A7r5 was investigated. Chronic depolarization of the cells by elevation of extracellular K^+ ($[K^+]_o$) decreased L channel number, as measured by dihydropyridine binding to membrane preparations. Maximum changes (40%) were observed within 4 days, resulting in a new steady state channel number which returned to control in 3 days upon restoration of the normal $[K^+]_o$. Elevation of $[K^+]_o$ from 5 to 15 mM caused nearly maximum changes in channel number.

A7r5 cells displayed L channel dependent spontaneous action potentials. Action potential generation was unaffected by 15 mM $[K^+]_o$ but 50 mM $[K^+]_o$ caused an immediate, reversible cessation of spontaneous activity. Elevation of $[K^+]_o$ from 5 mM to 15 and 50 mM decreased the resting membrane potential from -55 mV to -40 and -17 mV, respectively. After 5 days of treatment with 15 mM $[K^+]_o$, maximum action potential rate of rise was decreased from 1.5 to 1.0 mV/s, suggesting that changes in channel number correlated with changes in this electrophysiologic parameter.

Since changes in Ca^{2+} channel activity could lead to alterations in intracellular Ca^{2+} ($[Ca^{2+}]_i$), the role of Ca^{2+} in controlling channel number was investiaged. Oscillations in $[Ca^{2+}]_i$ associated with the spontaneous action potentials were observed with fura-2. Chronic depolarization with 15 mM $[K^+]_o$ decreased average $[Ca^{2+}]_i$ from 142 to 107 nM. Chronic exposure of cells to reduced $[Ca^{2+}]_o$ caused a lowering in $[Ca^{2+}]_i$ and channel number to decrease in a time and $[Ca^{2+}]_o$ dependent manner. However, lowering $[Ca^{2+}]_o$ also depolarized the cells making it difficult to differentiate between the effects of altered membrane potential and decreased $[Ca^{2+}]_i$. These results indicate that L channel number in A7r5 cells may be regulated by factors controlling channel fuction (e.g. membrane potential) and/or reflecting channel activity (e.g. $[Ca^{2+}]_i$).

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Tu-Pos105

ROLE OF INTRACELLULAR Ca^{2+} STORES IN THE REGULATION OF THE PLASMA MEMBRANE Ca^{2+} PERMEABILITY OF RAT LYMPHOCYTES. M.J. Mason[1], M.P. Mahaut-Smith[2] & S. Grinstein[1].[1]Division of Cell Biology, Hospital for Sick Children, Toronto, Canada M5G 1X8 and [2]Cellular and Molecular Medicine M-047, University of California at San Diego, La Jolla, CA.

While it is well documented that mitogenic stimuli activate a membrane potential-sensitive Ca^{2+} permeability in T lymphocytes, little is known about the mechanism(s) responsible for Ca^{2+} uptake into unstimulated cells. Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is stringently controlled in resting cells, suggesting the existence of a $[Ca^{2+}]_i$-sensitive pathway. To test this hypothesis we depleted rat thymic lymphocytes of internal Ca^{2+} by exposure to Ca^{2+} -free solution and trapping of intracellular Ca^{2+} chelators. Upon readdition of Ca^{2+} to the extracellular medium, normal $[Ca^{2+}]_i$ was rapidly restored, as measured by the fluorescence of the indicator indo-1. During the $[Ca^{2+}]_i$ recovery phase, the rate of unidirectional $^{45}Ca^{2+}$ uptake was increased 8-fold compared to non-depleted cells. As in mitogen (concanavalin A)-stimulated cells, the $[Ca^{2+}]_i$ recovery following depletion was inhibited by depolarization, by La^{3+} and by compound SK&F 96365, a novel blocker of receptor-mediated Ca^{2+} entry. $^{45}Ca^{2+}$ uptake was similarly affected by these agents and conditions. Comparison of the time course of $[Ca^{2+}]_i$ recovery and of the rate of $^{45}Ca^{2+}$ influx revealed that the Ca^{2+} entry pathway remains activated for some time after normal $[Ca^{2+}]_i$ is regained. This observation suggested that the rate of influx is controlled by a parameter other than $[Ca^{2+}]_i$, possibly the degree of filling of intracellular Ca^{2+} stores. Consistent with this notion, depletion of internal stores with thapsigargin, a naturally occurring inhibitor of the Ca^{2+}-ATPase of endomembranes, also elevated $[Ca^{2+}]_i$ and the rate of $^{45}Ca^{2+}$ uptake across the plasma membrane. Ca^{2+} influx induced by thapsigargin was similarly sensitive to depolarization and was blocked by La^{3+} and by SK&F 96365. The magnitude of the intracellular Ca^{2+} stores was estimated measuring $[Ca^{2+}]_i$ fluorimetrically in Ca^{2+}-free solutions. As expected, thapsigargin greatly depleted the internal stores. More importantly, substantial reduction in the size of the stores was also found following the $[Ca^{2+}]_i$ depletion protocol or the addition of concanavalin A. The data are consistent with the hypothesis that the degree of filling of an intracellular Ca^{2+} pool regulates the plasma membrane Ca^{2+} permeability. This mechanism is involved in the agonist-induced increase in $[Ca^{2+}]_i$ and also in the control of $[Ca^{2+}]_i$ in unstimulated cells.

Tu-Pos106

TWO COMPONENTS OF VOLTAGE-GATED CALCIUM CURRENT IN CIRCULAR SMOOTH MUSCLE CELLS OF COLON. Jacob Krier and David R. Bielefeld, Department of Physiology, Michigan State University, East Lansing, MI 48824

Experiments were designed to determine whether different populations of calcium channels exist for circular smooth muscle of the cat colon. Macroscopic inward currents in calcium (2.0-20 mM) and barium (20 mM) were recorded at room temperature (22-25°C) from single isolated circular smooth muscle cells of cat proximal colon using the whole-cell patch clamp technique. Cells were dialyzed with Cs^+ and an external Cs^+-TEA solution was used to suppress K^+ currents. Two distinct components of the macroscopic calcium or barium current could be resolved: a transient low threshold (T-type) current and a sustained, high threshold (L-type) current. Co^{2+} (2 mM) blocked all inward current components to the same degree. Tetrodotoxin (10 mM) did not block or reduce either component. The T-type current was recorded only when cells were held at very negative potentials (-100 mV, -90 mV), activated positive to -60 mV to -50 mV, peaked at -20 mV to -10 mV, prominent at negative depolarizations and reversed at approximately +20 mV to +30 mV. This current was insensitive to nifedipine (0.5 mM) and BAY K 8644 (1 mM), but blocked selectively by 40 uM to 100 uM Ni^{2+}. The T-type current half maximal activation was -34.1 mV in 2.0 mM Ca^{2+} and -23.2 mV in 20 mM Ca^{2+}. The L-type current was recorded at less negative holding potentials (-40 mV). It activated positive to -20 mV to -10 mV, peaked at +10 to +20 mV and reversed at approximately 50 to 60 mV. It was prominent at positive depolarizations, enhanced by BAY K 8644 (1 mM), blocked by low concentrations of nifedipine (0.5 mM), unaltered by 40 to 50 μM Ni^{2+} and minimally reduced by the presence of 100 uM Ni^{2+}. The L-type current half maximal activation was -11.7 mV in 2.0 mM Ca^{2+} and 4.8 mV in 20 mM Ca^{2+}. Steady-state inactivation for the T- and L- type current was half maximal at -51.0 mV and -32.0 mV, respectively in 2.0 mM Ca^{2+} (NIADDK- 29920).

Tu-Pos107

KINETICS OF OPEN CHANNEL BLOCK OF SINGLE $GABA_A$ RECEPTOR CHANNELS BY PENICILLIN

R.E. Twyman*, R.M. Green* and R.L. Macdonald*#
Deptarments of Neurology* and Physiology#
University of Michigan, Ann Arbor, MI 48104.

Penicillin (PCN) reduces GABA-evoked chloride currents and has been shown to produce myoclonus and seizures when administered *in vivo* at sufficiently high doses. PCN reduction of GABA-evoked current has been suggested to occur by open channel block or allosteric modulation of the GABA receptor. To determine the mechanism of action of PCN on $GABA_A$ receptors, the kinetics of PCN reduction of single GABA receptor currents recorded from excised outside-out patches of mouse spinal neurons in culture were analyzed.

In patches clamped at -75 mV in symmetrical chloride solutions, GABA (2 mM) evoked inward currents that were reduced in the presence of PCN (100-5000 μM). Although channel conductance was not altered, there was a concentration-dependent decrease in GABA-evoked current, mean channel open time and in the time constants obtained from open duration frequency histograms. Opening frequency, mean burst durations and openings per burst increased with PCN concentration.

The concentration-dependent alteration in the kinetics of GABA-evoked currents by PCN can be explained by a simple open channel block mechanism. However, the data also provided evidence for blocker association and dissociation rates that were dependent on the stability of the channel open states. A microscopic kinetic reaction scheme consistent with the results is presented. Channel activity predicted by the reaction scheme was simulated and compared well with the experimental results. The significance of state dependent block is discussed.

Tu-Pos108

CA^{2+}-ACTIVATED K^+ CHANNELS IN GH_4 CELLS ARE STIMULATED BY SOMATOSTATIN AND INHIBITED BY cAMP-DEPENDENT PHOSPHORYLATION. Richard E. White and David L. Armstrong, Laboratory of Cellular and Molecular Pharmacology, NIEHS, Research Triangle Park, North Carolina

In rat pituitary tumor cells, the neuropeptide somatostatin (SST) inhibits secretion through two pertussis toxin-sensitive mechanisms: i) inhibition of adenylate cyclase and ii) an unidentified cAMP-independent process that also modulates voltage-activated Ca^{2+} and K^+ channels (Luini et al. '86, *J.Neurosci.* 6:3128; Koch et al. '88, *J.Biol.Chem.* 263:216; Mollard et al. '88, *Endocrinol.* 123:721). We have reexamined the regulation of ionic currents by SST in *metabolically-intact* GH_4 cells that were bathed in physiological saline augmented with 1 μM tetrodotoxin and voltage-clamped through membrane patches permeabilized with nystatin (Horn & Marty '88, *J.Gen.Physiol.* 92:145). SST decreased peak I_{Ca} and increased steady-state outward current at all voltages. Maximal effects were obtained with 100 nM SST which depressed I_{Ca} by 28 $\pm$ 2% (at +10 mV) and increased steady-state outward current by 91 $\pm$ 16% (at +30 mV). In contrast, stimulation of adenylate cyclase with maximal concentrations of vasoactive intestinal peptide [100 nM] or forskolin [10 μM] inhibited the steady-state outward current in intact cells, as did bath application of 2 mM dibutyryl cAMP. This current was predominantly I_{KCa} because it was inhibited completely by replacing K^+ in the pipet with Cs^+ and inhibited > 80% by adding 2 mM Co^{2+} or 1 mM TEA^+ to the bath. Single channel records from cell-attached and cell-free patches in normal K^+ gradients confirmed that identification. In cell-attached patches, adding SST to the bath *outside* the patch pipet rapidly stimulated the activity of a TEA-sensitive, 120 pS channel with an extrapolated reversal potential near E_K. In outside-out patches this channel was also inhibited by 2 mM dibutyryl cAMP in an ATP-dependent manner. In some cells and patches, but not all, subsequent addition of 100 nM SST overcame the inhibition by dibutyryl cAMP. These results suggest that SST increases the activity of Ca^{2+}-activated K channels in GH_4 cells by stimulating a phosphatase to dephosphorylate the channel protein or a closely associated regulatory molecule.

Tu-Pos109

ROLE OF ION CHANNELS IN CELL VOLUME REGULATION IN N1E115 NEUROBLASTOMA CELLS. Roger Yang, Lee Falke, and Stanley Misler, The Jewish Hospital, St. Louis, MO 63110.

N1E115 cells exposed to hypotonic solutions (low π) initially swell and then undergo a regulatory volume decrease (RVD) even in low Ca^{2+}_o. Based on cell-attached patch recording during swelling and RVD we proposed that the sequential opening of three ion channels, whose activities were not well modulated by cytosolic Ca^{2+}, might underlie RVD. That is: (1) Rapid cell swelling --> opening of stretch activated non-selective cation channels, [C^+(SA)]. (2) The resultant membrane depolarization --> opening of voltage dependent delayed rectifier type K^+ channels, [K^+(DR)], and voltage dependent large conductance anion channels, [A^-(V)]. (3) Opening of the latter two channels --> exit of K^+ salts and water with cell shrinkage and membrane repolarization.

Several new lines of evidence support this hypothesis. (1) During conventional whole cell recording, cells exposed to a reduction in π from 325 to 230 mOsm rapidly swelled; depolarized from rest V_m of -60 to -45 mV to near 0 mV; and increased their membrane conductance (g_m) nearly 15 fold. They nearly doubled their cross-sectional area (csa) but displayed neither RVD nor repolarization. (2) In contrast, during perforated patch recording, (access resistance 80-100 MΩ), similarly exposed cells initially swelled to 1.3 - 1.6 their original csa over 4-6 min. and then underwent RVD over the next 20-30 min. Cells depolarized to -45 to -15 mV. g_m peaked at 10-15 times control value but usually after the peak of V_m. V_m and g_m recovered towards baseline during RVD. (3) RVD was abolished by replacing $NaCl_o$ with KCl_o, and after addition of the stilbene DIDS or gadolinium, which block similar A^-(V) and C^+(SA) channels, respectively, in other cells. Findings 1-3 are consistent with the early opening of C^+(SA) channels and subsequent opening of other opposing channels when RVD is intact. (4) Neither swelling nor RVD was accompanied by significant changes in cytosolic Ca^{2+}, as determined in FURA-2 loaded cells. (5) Changes in csa reflected three-dimensional changes in cell geometry seen with confocal microscopy of dil stained cells. Support: PHS NS 27983.

Tu-Pos110

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF A HYPOTHALAMIC NEURONAL CELL LINE.
Martha M. Bosma and Bertil Hille. Dept. of Physiology and Biophysics, Univ. of Washington, Seattle, WA 98195.
We have used tight-seal whole-cell recording, both in voltage and current clamp modes, to characterize a unique neurosecretory cell line of CNS origin. This cell line was derived from gonadotropin-releasing hormone (GnRH) secreting cells by fusing the GnRH promoter gene to the SV40 T-antigen oncogene, and making transgenic mice, some of which consequently had hypothalamic tumours from which the cell lines were cultured (Mellon et al., Neuron 5:1-10, 1990). Depolarizing steps elicit fast inward Na current, two components of inward currents in Ca channels, and three outward K currents. Hyperpolarization activates an inward rectifier K current at potentials negative to -60 mV. In addition, GABA opens $GABA_A$ receptor channels. The Na current is completely blocked by 10 μM TTX. In 15 and 30 mM Ba solutions, currents in Ca channels have transient and sustained components. The transient current ranges between 0-15% of the total, and is stable for the duration of the recordings (10-35 min). The sustained component is more substantial but labile and may run down 50% in about 3 min, although the pipette contains 3 mM Mg-ATP and leupeptin (0 Ca, 10 EGTA). The sustained current peaks at +30 to +40 mV in 15 Ba. The outward K currents differ in kinetics (one sustained and two transient currents), pharmacology, dependence on holding potential, and contribution of each type seen in each cell. None is dependent on Ca, since removing external Ca, adding external Cd or chelating internal Ca with EGTA makes little difference to any of the currents. The inward rectifier K current is completely blocked by 100 μM external Ba, and is suppressed about 50% with 100-200 nM GnRH.
Other laboratories have shown that these cells secrete GnRH in a periodic manner as they do in the intact animal (intervals of 20-90 min). Using nystatin-perforated patches and long-term current clamp recording, we have observed relatively quiescent cells (isolated from any neighboring cells) begin to fire bursts of action potentials, perhaps a direct cause or consequence of the secretion of GnRH in a periodic manner. The mechanism of these changes in activity is under investigation.
Supported by NS08174 and a McKnight Award to BH, training grant NS07097 and fellowship NS08868 to MB.

Tu-Pos111

POSITIVE INOTROPIC EFFECT OF ADRENALINE ON POTASSIUM CONTRACTURES IN SLOW SKELETAL MUSCLE FIBERS OF THE FROG.
J. Escamilla-Sánchez and M.C. García. Dept. of Physiology and Biophysics, ENCB-IPN. Mexico City.
Catecholamines potentiate twitch tension due to modulation of calcium channels via cAMP (Arreola et al., J. Physiol. (1987) 393: 307-330). In the present experiments we describe that adrenaline also increases the amplitude of the K^+ contractures of slow muscle fibers. Bundles of 4-6 slow fibers from semitendinous or tibialis anterior muscles of Rana pipiens were used for isometric tension measurements (RCA-5734) at 20-22°C. Potassium contractures were elicited by solutions with constant $[K^+]_o \times [Cl^-]_o$. When high $[Ca^{2+}]_o$ was added, $CH_3SO_3^-$ was used instead of $SO_4^{=}$ as anion. Adrenaline (10^{-6}M) increased the amplitude of potassium contractures in a $[K^+]_o$ dependent manner: with 20 mM $[K^+]_o$ the slow component of contractures (measured at 1 min. from the onset) increased 38.6 ± 8.3% (n = 7); whereas the peak amplitude increased 26.4 ± 7.7% (n = 5), these effects were less prominent at large $[K^+]_o$. Increasing $[Ca^{2+}]_o$ from 1.8 to 10 mM practically doubled the positive inotropic effect of adrenaline (n = 21). In contrast, adrenaline had no effect after complete replacement of Ca^{2+} by Mg^{2+} suggesting a role for Ca^{2+} channels. In agreement with this possibility, Nifedipine (20 μM) reversibly inhibited the positive inotropic effect of adrenaline (n = 3). Membrane potential was unaffected by adrenaline at all $[K^+]_o$ (n = 54). In conclusion, adrenaline has a potentiating effect in slow muscle fibers especially in the slow component of potassium contractures. This effect depends on external Ca^{2+} and may be mediated through calcium channels.

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Tu-Pos112

Regulation of the Dihydropyridine Receptor Expression by Biomechanical Unloading.

S. O'Brien, K. Thomas, L. Schulte, H. Lucero, S. Kandarian and J. Navarro. Boston University School of Medicine. Departments of Physiology and Biochemistry. Boston, MA 02118. Sargent College Department of Health Sciences. Boston, MA 02215.

Biomechanical unloading of the rat soleus by hindlimb unweighting (HU) is known to induce atrophy and slow to fast transition of many skeletal muscle properties. For example, we have shown a 37% reduction in total contraction time in the unloaded soleus. In addition, the expression of the dihydropyridine (DHP) receptor appears to be related to fiber type. Therefore, we analyzed the expression of the DHP receptor gene in unloaded fast and slow twitch muscles. We isolated the cDNA encoding the rat DHP receptor by screening a random primed cDNA λgt10 library from denervated rat skeletal muscle with oligonucleotide probes deduced from the coding region of the rabbit DHP receptor cDNA. A clone 1Fb was sequenced and revealed a high degree of homology to the rabbit cDNA. Northern blot analysis showed that tissue distribution of DHP receptor mRNA was specific for skeletal muscle and expression was several fold higher in fast twitch than slow twitch muscle. Biomechanical unloading for 28 days induced marked atrophy and increased expression of the DHP receptor mRNA in soleus approaching levels normally expressed in fast twitch muscles. Time course studies indicated a significant stimulation in receptor message after 24h of HU without changes in muscle mass. Our results suggest that muscle activity necessary for weight support modulates the expression of the DHP receptor gene.

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Tu-Pos113

DEPENDENCE OF FREE CALCIUM CONCENTRATION ON CALCIUM CURRENT IN CANINE GASTRIC SMOOTH MUSCLE CELLS.
F. Vogalis, N. Publicover and K.M. Sanders. Dept. Physiology, University of Nevada, Reno, NV 89557, USA.
Canine antral circular muscle generates slow waves and contractions that are sensitive to Ca-channel blockers and manipulations in $[Ca^{2+}]_o$. The relationship between Ca^{2+} influx through voltage-gated Ca-channels (I_{Ca}) and intracellular free calcium concentration ($[Ca^{2+}]_i$) was investigated in voltage-clamped, Cs^+- and INDO-1-loaded antral myocytes. $[Ca^{2+}]_i$ was measured simultaneously with I_{Ca} using a ratiometric fluorescence method. Cells were bathed in a HEPES buffered solution containing 3.6 mM Ca^{2+}. The resting $[Ca^{2+}]_i$ averaged 144 ± 20 nM (n=11) at a holding potential of -70 mV and increased commensurately with I_{Ca} at potentials positive to -50 mV. Peak I_{Ca} and $[Ca^{2+}]_i$ occurred in the same range (0 to +10 mV) and averaged 315 ± 51 pA and 372 ± 48 nM (n=8), respectively. Depolarizations mimicking electrical slow waves (i.e. upstroke and plateau phases) of intact muscles elicited a transient I_{Ca} followed by a sustained I_{Ca}. The transient I_{Ca} caused a small increase in $[Ca^{2+}]_i$, and the sustained I_{Ca} caused a slow accumulation of $[Ca^{2+}]_i$ with plateau depolarizations positive to -40 mV. This secondary increase in $[Ca^{2+}]_i$ reached 300 nM after a 7-s depolarization to -35 mV. We suggest that the second phase accumulation of $[Ca^{2+}]_i$, which is dependent upon the magnitude of the sustained I_{Ca}, is the basis for the "mechanical threshold" in antral muscles. The decrease in $[Ca^{2+}]_i$ following repolarization was slow (time constant averaged 1.4 s). During the period that $[Ca^{2+}]_i$ decreased, I_{Ca} was partially inactivated, and the recovery of I_{Ca} from inactivation paralleled the decrease in $[Ca^{2+}]_i$. These data suggest that the rise in $[Ca^{2+}]_i$ during slow wave depolarizations is dependent upon I_{Ca} and $[Ca^{2+}]_i$ may regulate the availability of Ca^{2+} channels in antral smooth muscle. (Supported by DK 32176 and DK 40569)

Tu-Pos114

ION FLOW THROUGH MEMBRANES AND THE RESTING POTENTIAL: A MORE ACCURATE FORMULATION. Franklin Offner, Biomedical Engineering Dept., Northwestern University, Evanston, IL 60208

The relationship between ion flow (both active and passive), ionic concentration, and membrane potential, is essential to the understanding of cellular function. The Goldman (and thus the GHK) equation suffers from its long-recognized approximations; while the chord-conductance approach does not include concentration effects. It is shown that at low concentrations and current flow I_J of species J, permeability P_J, is proportional to the sum (or average) of the internal and external concentrations: $I_J = P_J(V-E_J)(C_{Jint}+C_{Jext})$; E_J is the equilibrium voltage, and V is the actual voltage across the membrane. At higher concentrations, the concentrations are corrected for saturation by multiplying by the factor $C_{J/h}/(C_{J/h}+C_{Jint})$ [or C_{Jext}]; $C_{J/h}$ is the concentration of half-maximum conductance. $C_{J/h}$ will increase proportional to I_J at the interface at which ions enter the channel, and decrease at the other. Similarly, if there are other cation species in the bath, C_J will decrease in the immediate vicinity of the mouth of the channel at which ions enter, proportionate to I_J and the mole-fraction of competing anions; the converse occurs at the other mouth. The net effect will be a change in the value of E_J. These factors will result in a non-linear I-V relationship.

The equation for the resting potential V_0, including the effect of Na,K-ATPase and assuming only Na^+ and K^+ flow, is then $V_0 = (G_{Na}E_{Na} + r \cdot G_K E_K)/(G_{Na} + r \cdot G_K)$; r is the Na:K transport ratio, and $G_J = I_J/(V-E_J)$, where I_J is calculated using the relationships discussed above. Since at rest I_J is small, nonlinearity effects may be neglected.

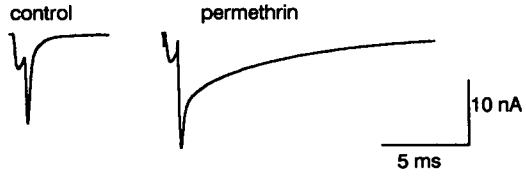
A similar analysis is given for the potential of epithelial cells; this has resolved the long-standing problem of the source of the positive potential of the scala media of the cochlea.

Comparisons of the predictions of the above relationships with available experimental data are presented, and compared with earlier formulations; a protocol for further experimental test is suggested.

Tu-Pos115

PERMETHRIN PROLONGS SODIUM CHANNEL TAIL CURRENTS IN TROUT SENSORY NEURONS. T.M. Frank and D.T. Campbell. Hatfield Marine Science Center, Oregon State University, Newport, OR.

Biochemical and behavioral assays demonstrate that fish are significantly more sensitive than mammals to pyrethroid insecticides. Pyrethroids bind to Na channels, prolonging channel closing. Thus the greater toxicity to fish may be due, in part, to a higher sensitivity of fish Na channels to pyrethroids. The effects of pyrethroids on mammalian Na channels have been studied extensively, but similar studies on fish have been hindered by the lack of a suitable voltage clamp preparation. We have developed a trout sensory neuron preparation for studying Na channels using the whole cell patch clamp method. Nerve cell bodies are enzymatically dissociated from trout nodose and petrosal ganglia and maintained in Eagle's MEM + 10% FBS. Na currents from trout sensory neurons exhibit kinetics and TTX sensitivity similar to those measured in other vertebrate neurons. We have begun studying the effects of the pyrethroid permethrin, which has been shown to be 20-100 times more toxic to fish than to mammals. Permethrin (10 μM) greatly prolongs the decay of Na tail currents elicited by a return to -80 mV from a prepulse of 10 mV (figure). A smaller but significant effect was produced by 1 μM, whereas 100 nM produced an effect too small to quantify. Thus, qualitatively, permethrin prolongs Na channel closing in a manner similar to that described in mammalian studies using other pyrethroids. Supported by USPHS grants NS22577 and ES07060.



Tu-Pos116

TITRATING THE CONDUCTANCE OF BTX-MODIFIED BRAIN SODIUM CHANNEL.
P. DAUMAS and O.S. ANDERSEN. Dept. of Physiology. Cornell University Medical College. New York, NY 10021.

We have examined the effects of symmetrical and asymmetrical pH changes on lipid bilayer-incorporated batrachotoxin (BTX)-modified sodium channels. In 1.0 M NaCl, when the pH is lowered symmetrically (from 7.4 to 4.9), the small signal single-channel conductance (g) decreases from 32 to 14 pS. Concommitant with this decrease in conductance, the current-voltage characteristics are rectifying (at pH < 5.5). The results for the small signal conductance cannot be fitted by a single-site titration curve, which implies that the conductance is modified by the titration of more than a single site. In order to adress this question, we turned to asymmetrical pH change experiments. In 1.0 M NaCl, when only the extracellular pH is decreased, the current-voltage characteristics begin to display rectification at pH < 6.0, but the conductance decreases are less than those seen with symmetrical conditions. The interpretation of these results is presently affected by H^+ permeation through the channel. But with that provision, the results are consistent with the notion that there may be two seperate titration sites, one accessible from the intracellular and one accessible from the extracellular solution.

Tu-Pos117

GRAYANOTOXIN MODIFICATION OF PURIFIED EEL ELECTROPLAX SODIUM CHANNELS IN PLANAR BILAYERS.
D.S. Duch[1], S.R. Levinson[2], and B.W. Urban[1,3], Dept. of Anesthesiology, [1]Cornell University Medical College, N.Y., N.Y., Dept. of Physiology, [2]University of Colorado Health Sciences Center, Denver, CO., and [3]Institut für Anästhesiologie, Universität Bonn, Bonn, FRG.

The alkaloid toxins, binding competitively to the voltage-sensitive sodium channel, have many similar yet distinct effects on sodium channel function. We have previously reported the properties of purified electroplax sodium channels in the presence of batrachotoxin (BTX; Recio-Pinto et al., J. Gen. Phys., 90, 375, 1990) and veratridine (Duch et al., J. Gen. Phys., 94, 813, 1989). In the presence of another related toxin of this group, grayanotoxin (GTX), eel sodium channels had a linear I-V relationship (symmetrical 500 mM NaCl, pH 7.4) between ±100 mV, similar to both BTX and veratridine. The slope conductance was 15pS, however, smaller than with BTX (25 pS) but larger than with veratridine (10-12 pS). The voltage-dependence of GTX-modified channel open times showed similarities with both BTX and veratridine-modified channels. Activation gating similar to BTX-modified channels, but not detected with veratridine, was observed with a midpoint (Va) of -90 mV, about 20 mV more negative than with BTX. In addition, a superimposed shallow voltage-dependence of channel open times previously found with veratridine, but absent with BTX, was found with GTX. This shallow voltage-relationship was also shifted about 20 mV more negative than with veratridine. These results indicate that GTX-modified channels have properties intermediate between veratridine and BTX, and thus become a useful tool for understanding toxin modification of the sodium channel at this site.

Tu-Pos118

PRESENCE OF EARLY EMBRYONIC SLOW Na^+ CHANNELS IN CARDIOMYOPATHIC HAMSTER.
D. Jacques and G. Bkaily, Dept. of Physiology and Biophysics, Fac. Medicine, Univ. of Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4.

We have recently demonstrated the presence of a slow inward Na^+ current (TTX- and Mn^{2+}-insensitive) in early embryonic chick heart cells. This slow Na^+ channel is permeable to Na^+, Li^+, Ba^{2+}, but not permeable to Ca^{2+}. This channel is highly sensitive to melitin and apamin and relatively sensitive to organic Ca^{2+} blockers. The objective of this study is to verify the hypothesis in that the slow Na^+ channel may continue to function during development of the heart of cardiomyopathic hamster.

In order to test our hypothesis, single cells were isolated from normal and cardiomyopathic hamster and inward currents were recorded using the whole-cell voltage clamp technique. The inward current of the normal heart cells was TTX sensitive and behaved as was expected for a classical fast Na^+ current. However, the inward current recorded from cells of the cardiomyopathic hamster was insensitive to TTX and Mn^{2+} and had characteristics similar to the Na^+ channel in early embryonic hearts. These results suggest that the TTX- and Mn^{2+}-insensitive I_{Na} exist in cardiomyopathic cells and this current may contribute to the increase of $[Na]_i$; thereby, allowing Ca^{2+} to flow in through the Na-Ca exchanger.

This research is supported by MRCC MT9816 and Dr Bkaily is Merck Frosst-FRSQ Professor and D. Jacques is a fellow of CHF.

Tu-Pos119

TETRODOTOXIN BLOCK OF HUMAN ATRIAL SODIUM CURRENT.

Y Sakakibara, JA Wasserstrom, T Furukawa, H Jia, SL Eager, CE Arentzen, CL Backer, DH Singer (Intro. by BF Hoffman)
Reingold ECG Center, Northwestern University, Chicago, IL.

Although much is known about the characteristics of tetrodotoxin (TTX) block of cardiac Na^+ current (I_{Na}) in animal models, little is known about its effects in human heart. We studied TTX block of I_{Na} in isolated human atrial myocytes at 17°C using whole cell voltage clamp methods. Normal appearing rod-shaped, striated, Ca^{2+} tolerant cells were isolated enzymatically from atrial appendage specimens obtained from 44 patients undergoing cardiac surgery. K^+ and Ca^{2+} currents were blocked by Cs^+ outside and F^- inside, respectively. Both external and internal solutions contained 5mM Na^+. TTX reduced I_{Na} in a dose-dependent manner. The TTX dose-response curve was fitted by a sigmoid curve, indicative of 1:1 binding of TTX to Na^+ channels with resting state Kd=1.7μM (n=8). TTX did not shift the steady-state availability curve. The magnitude of TTX block was not affected by a change in holding potential (V_h) to -100mV, where more than 30% of I_{Na} was inactivated. TTX did not affect the rate of I_{Na} inactivation. TTX produced use-dependent block (UDB) during pulse trains with an inter-pulse interval of 500ms (n=10). UDB occurred at pulse durations as short as 3ms suggesting, that TTX binds to the activated state. Changes in V_h did not influence UDB. Recovery from inactivation was biphasic under control conditions, with an initial fast phase (τ_{fast}=6.5±0.5ms, mean±S.E.) followed by a slow phase (τ_{slow}=66.3±8.9ms, n=6). TTX did not affect τ_{fast} but increased τ_{slow} significantly to 2025±144ms. Changing V_h did not affect τ_{slow} between -140 and -90mV. Finally, the onset of TTX block showed both a rapid phase and a slow phase (n=6). We conclude that 1) TTX sensitivity of I_{Na} in human atrial cells is similar to that for other mammalian species; 2) inactivated state Kd for TTX is similar to that for resting state; 3) TTX also binds to the activated state and dissociation from this state is slow; 4) both binding to and dissociation from the inactivated state are very slow; 5) this probably underlies TTX induced UDB.

Tu-Pos120

FUNCTIONAL EXPRESSION OF SkM2, A TTX-INSENSITIVE SODIUM CHANNEL CLONED FROM DENERVATED SKELETAL MUSCLE.

M. White, L. Chen, R. Kallen, and R. Barchi, University of Pennsylvania, Philadelphia PA.

We recently cloned and characterized a sodium channel (SkM2) from rat skeletal muscle which exhibits a pattern of steady-state mRNA expression corresponding to that expected for the TTX-insensitive (TTX-I) sodium channel in denervated and cardiac muscle (Kallen et al., Neuron 4:233-242, 1990). The sequence of this channel is identical to one cloned from rat cardiac muscle by Rogart et al. (PNAS 86:8170-8174, 1989). We constructed a full-length clone of the SkM2 channel that included the entire open reading frame, 206 nucleotides of 5' untranslated region (5'UT) and 816 nucleotides of the 3'UT from several overlapping partial clones and subcloned it into the pSP64T vector. Two additional constructs were then made with shorter 5'-UT of 17(D3-2) or 4(D3-48) nucleotides. Capped mRNAs were synthesized in vitro from each of the three constructs and injected into stage V and VI Xenopus oocytes.

All three constructs gave rise to voltage-dependent transient inward currents in response to voltage jumps from a holding potential of -100 mV to -20 mV, but the size of the current was highly dependent on the length of the 5'UT. When equal amounts of RNA were injected, mRNA from the construct with the 4 nucleotide 5'UT (D3-48) produced currents 7-fold greater than the mRNA with the 206 n 5'UT although both constructs supported the synthesis of appropriately sized transcripts. For D3-48 mRNA, currents reached a maximum for depolarizations to -20 mV. Currents activated and inactivated rapidly, with a voltage-dependent time constant for inactivation (τ_h) ranging between 5.2 +/- 0.1 msec at -40 mV and 2.7 +/- 0.2 msec at -10 mV. The relationship of h_∞ vs. V, measured by depolarization to -20 mV after 50 msec prepulses, was described by a Boltzman distribution with a midpoint at -66 mV and a slope factor of k = 7.4 mV. The expressed currents were highly resistant to 100 nM TTX. A complete dose-response curve for TTX indicated a K_d = 1.9 uM. The currents were also unaffected by exposure to 100 nM u-conotoxin.

These data confirm that the sodium channel encoded by the SkM2 cDNA is the TTX-resistant form of the sodium channel found in denervated muscle and heart.

This work was supported by NIH grants NS-18013 and NS-23885.

Tu-Pos121

IDENTIFICATION AND CHARACTERIZATION OF THREE DISTINCT ACTIVATION MODES IN BTX-MODIFIED SODIUM CHANNELS.

A. M. O'Connell and O. S. Andersen, Cornell Univ. Med. Coll., New York, N.Y.

At any given membrane potential (Vm), spontaneous reversible shifts in the activation of single BTX-modified sodium channels result in a clumping of open probability (Po) values, separated by regions rarely if ever visited. This grouping is evident in sodium channels from rat brain incorporated into planar lipid bilayers under a variety of membrane and electrolyte compositions, and does not show any obvious voltage- or history-dependence. At least three distinct modes that do not differ grossly in kinetics or conductance can be identified on the basis of this clumping. That is, a mode shift is not accompanied by any dramatic change in the pattern of channel openings. For individual channels, there is a slight (2-3%) decrease in the open channel conductance going from mode I to mode II and again from mode II to mode III, but this difference is not significant when conductance values for all the channels are pooled. A single channel can visit all three of the modes reversibly, and only a few channels were not observed in at least two different modes. (These channels may not have been observed long enough for mode changes to occur.) The overall percent of time spent by the channels in each of these three modes decreases as the midpoint potential of activation ($V_{\frac{1}{2}}$) becomes less negative, from 67% (mode I) to 29% (mode II) to 1% (mode III), indicating that there is an energetic cost associated with the conformational changes that result in a depolarizing shift in activation. The apparent gating valence, z, decreases as $|V_{\frac{1}{2}}|$ decreases (r=.69), averaging 3.6 for channels in mode I ($V_{\frac{1}{2}} \approx -86$ mV), 3.0 for mode II ($V_{\frac{1}{2}} \approx -74$ mV) and 1.9 for mode III ($V_{\frac{1}{2}} \approx -56$ mV). These results demonstrate that the variability in voltage-activation of voltage-dependent sodium channels does not result from the existence of a continuum of gating states, but rather from transitions among a finite number of distinct gating modes.

Tu-Pos122

SLOW INACTIVATION OF SCHWANN CELL SODIUM CHANNELS.

J.R. Howe & J.M. Ritchie. Dept. of Pharmacology, Yale University School of Medicine, New Haven, CT 06510.

Schwann cells cultured from neonatal and adult rabbits express voltage-gated Hodgkin-Huxley type sodium channels (Chiu *et al.*, 1984; Howe & Ritchie, 1990). We have studied the kinetics of inactivation of sodium currents evoked in these cells with patch-clamp recording techniques. The decay of whole-cell currents, or of ensemble currents obtained from outside-out patches, was reasonably well-described by single exponential fits which gave time constants, τ_h, similar to those found for such currents in nerve. Although inclusion of an additional exponential component usually improved the fits to the decay of these currents, the relative amplitude of the slower component (time constant 3 to 6 ms) was always small (< 5%). However, both the recovery from steady-state inactivation and also the onset of inactivation clearly contained two exponential components, one with a time constant on the order of seconds. At -120 mV, recovery from steady-state inactivation at -50 mV (determined from changes in the amplitude of the peak sodium current as a function of prepulse duration) proceeded in two phases, one with a time constant of 2.24 ± 0.22 ms and one with a time constant of 1.16 ± 0.18 sec (n = 9). The relative amplitude of the slow component was 56.5 ± 2.8%. The corresponding amplitude of the slow component when similar experiments were done from holding potentials of 0 mV and -70 mV was 79.7 ± 4.2% and 36.2 ± 3.3%, respectively (n = 5 and 8). The time constant of the slow component of recovery from sodium-channel inactivation depended on membrane potential. It increased to 10.72 ± 0.98 sec at -70 mV (n = 6). The onset of steady-state inactivation also followed a bi-exponential time course. The time constant of the slower component was similar at each potential examined (0, -50, and -70 mV), the mean value being 6.65 ± 0.52 sec (n = 31).

Records of single-channel sodium currents that were evoked by a series of test depolarizations tended to cluster into records which did, and records which did not, contain openings. This non-random behaviour depended on membrane potential, and on the frequency at which the test steps were repeated, in the way expected if this non-randomness was the result of slow inactivation. Although prolonged membrane hyperpolarization increased the amplitude of ensemble current records, it did not alter their time course.

Our results indicate that sodium channels in rabbit Schwann cells are subject to a type of inactivation that is only removed by prolonged membrane hyperpolarization to potentials in excess of -100 mV.

Supported by grants NS12327 (USPHS) and RG1125 (MS Society).

Chiu, S.-Y., Shrager, P. & Ritchie, J.M. (1984). *Nature* 311, 156-157.
Howe, J.R. & Ritchie, J.M. (1990). *J. Physiol. (Lond.)* 425, 169-210.

Tu-Pos123

TRIMETHYLOXONIUM MODIFICATION OF BATRACHOTOXIN-ACTIVATED SODIUM CHANNELS WEAKENS μ-CONOTOXIN BLOCK. D.B. Cherbavaz and C. Miller, Biophysics Program and Graduate Dept. of Biochemistry, Brandeis Univ., Waltham, MA 02254

To probe protein surface charge influencing toxin binding to batrachotoxin-activated sodium channels, the salt dependence of μ-conotoxin block was determined over a range of sodium concentrations. μ-ConotoxinGIIIA, a component of piscivorous marine snail venom, is a 22-amino acid peptide containing three internal disulfides and seven positively charged amino acids. Single sodium channels from rat skeletal muscle were incorporated into planar lipid bilayers and observed at symmetric sodium concentrations under voltage-clamp conditions. As is expected for a highly charged molecule, μ-conotoxin binding affinity is strongly dependent upon the salt concentration. The toxin affinity decreases more than two orders of magnitude as the sodium concentration is increased only one order of magnitude (from 10 to 100 mM). This effect is primarily due to a large decrease in the on-rate and is consistent with the presence of surface charge enhancing the local concentration of the highly charged toxin near the sodium ion channel protein. The off-rate increases weakly with salt concentration.

An alternate method to determine the effect of surface charge on protein function is to attempt to remove it. To this end, single sodium channels incorporated into planar lipid bilayers were exposed to trimethyloxonium (TMO) to convert carboxylate groups to uncharged methylesters. The affinity of μ-conotoxin for TMO modified Na channels is severly reduced, yet toxin binding remains strongly dependent on the salt concentration. Data will be quantitatively analysed to assess the action of TMO modification in removing surface charge near the toxin binding site.

Tu-Pos124

'AGONIST ' INDUCED SODIUM CHANNELS IN INSECT NEURONES: A KEY TO EVOLUTION OF THE SODIUM CONDUCTANCE? by Y.Pichon and M.Amar, Department of Biophysics, CNRS Laboratory of Cellular and Molecular Neurobiology, F-91190 Gif sur Yvette (France)

Amongst other molecules, the alkaloid veratridine and the insecticide deltamethrin have been shown to modify the properties of the sodium channels in several preparations. We have recently shown, that, in cultured insect neurones, the two Na channel 'agonists' were able to induce a voltage-dependent Na-conductance in embryonic cultured neurones which are normally inexcitable . The single channel events which underlie this conductance have been analyzed using the patch-clamp technique. With small concentrations of the 'agonists ' (in the micromolar range), the apparent open time probability was sufficiently low (between 0.001 and 0.01), to avoid superimposition of several channels, a necessary conditions for a proper analysis of the kinetics of the induced Na channels. Under those conditions, it was consistently found that the channels had complex kinetics which were similar, although not identical for veratridine and deltamethrin. Up to 10 subconductance levels could be observed in most patches, the mean conductance level (around 15-25 pS) corresponding to the addition of 3 to 4 levels. For both molecules, the open time at a given level could vary from a fraction of a ms up to several seconds and the distribution could not be fitted with a single exponential. The main difference between veratridine-induced and deltamethrin-induced Na channels was their 'bursting pattern'. Altogether, our results suggest that the primitive Na channel is made of a transient aggregation of a variable number of possibly identical subunits.

Tu-Pos125

USE DEPENDENT BLOCK BY TETRODOTOXIN OF RAT IIA SODIUM CHANNELS EXPRESSED IN *XENOPUS* OOCYTES. D.E. Patton and A.L. Goldin. Dept of Microbiology & Molecular Genetics, U. California, Irvine, CA 92717.

Most sodium channels of neuronal origin are blocked by nanomolar concentrations of tetrodotoxin (TTX). This is the case for sodium channels expressed in *Xenopus* oocytes following injection of RNA made *in vitro* from a rat brain cDNA clone termed rat IIA. It has recently been shown that block by TTX of sodium channels in frog myelinated nerve fibers is use dependent at hyperpolarizing holding potentials (U. Lönnendonker, 1989, Biochim. Biophys. Acta 985:153). We have found that block by TTX of rat IIA sodium channels expressed in *Xenopus* oocytes is also use dependent. To characterize this effect further, we examined block by 30 nM TTX using a two-pulse protocol with a two-electrode whole cell voltage clamp. The extra block (use dependence) increased with increasing time between pulses up to a maximum at 4 s and then decreased slowly, with full recovery at about 60 s between pulses. The voltage dependence of the extra block was examined by varying the potential of a 10 ms conditioning pulse which was followed 3 s later by a test pulse of constant amplitude. The amount of extra block increased with the level of depolarization of the conditioning pulse. To determine if this voltage dependence was related to activation we used a mutant (L860F) which displays a current-voltage relationship shifted in the hyperpolarizing direction. For this mutant the voltage dependence of the extra block was also shifted in the hyperpolarizing direction relative to wild-type. However, extra block developed at pre-pulse potentials that do not elicit significant activation in both the mutant and the wild-type. This suggests that the use dependence of TTX block of neuronal sodium channels may be due to a conformational change in the channel occurring during a voltage dependent transition prior to channel opening. To examine the effects of fast inactivation on the use dependent block we analyzed a linker insertion mutation which inactivates approximately 10-fold slower than wild-type. The characteristics of the use dependent block were similar in this mutant and wild-type, indicating that use dependent block is probably not related to fast inactivation.

Tu-Pos126

11-OXO TETRODOTOXIN, A POTENT ANALOGUE OF TETRODOTOXIN. **B. Q. Wu[1], L. Yang[1], C. Y. Kao[1], M. Yotsu[2] and T. Yasumoto[2]. [1]Department of Pharmacology, SUNY Downstate Medical Center, Brooklyn, NY 11203, USA, and [2]Faculty of Agriculture, Tohoku University, Sendai 981, Japan.**

11-oxo tetrodotoxin was discovered in the southern Pacific puffer fish, Arothron nigropunctatus. It has now been produced by oxidation of TTX with Fenton's reagent ($H_2O_2/FeSO_4$) and also in dimethylsulfoxide and 1,3-dicyclohexylcarbodiimide (DMSO/DCC). It differs from TTX in having the hydroxymethyl function on C-6 replaced by $CH(OH)_2$. TTX and 11-oxoTTX can be separated by HPLC. The relative potencies of TTX and of 11-oxoTTX in reducing sodium current has been determined on skeletal muscle fibers of the frog by the Hille-Campbell vaseline-gap method. At pH 7.25, the ED_{50} for TTX is ca. 4 nM. Under similar conditions, the ED_{50} for 11-oxoTTX is ca. 0.7 nM. Because the starting concentration of 11-oxoTTX was determined from the chromatogram, a maximum error of two fold may be allowed. Even with this allowance, 11-oxoTTX could be about 3 times more potent than TTX in blocking the sodium channel. The increased potency could be due to the presence of an additional -OH, which might enter into hydrogen-bonding with some channel amino acid residue. **(Supported by NIH grant NS 14551 and US Army contract DAMD17-87-C-7094).**

Tu-Pos127

TOXINS FROM LEIURUS QUINQUESTRIATUS HEBRAEUS ALTER & BLOCK Na CHANNELS. J. Borneman and R. Hahin, Biological Sciences Department, Northern Illinois University, DeKalb, IL 60114

Two protein toxins (Lqh1 and Lqh2) were purified from crude venom obtained from Middle Eastern scorpions, Leiurus quinquestriatus hebraeus by using cationic exchange chromatography. Lqh1 and Lqh2 were purified to homogeniety and possessed molecular weights of 6390 and 5870 Daltons respectively. Both toxins have smaller molecular weights than toxin V (7462) isolated from the North African scorpion, Leiurus quinquestriatus quinquestriatus. The application of each of these toxins to frog sciatic nerves showed that both Lqh1 and Lqh2 lengthened and attenuated compound action potentials recorded with the single sucrose gap technique. Dose response curves showed that both toxins are more potent than toxin V. Washout experiments designed to determine the relative rates of exit of the three toxins from their receptors, suggest that both Lqh1 and Lqh2 exhibit a reduced off-rate compared to toxin V.

Voltage-clamp experiments using the vaseline-gap technique and frog skeletal muscle fibers showed that Lqh1 acts to block sodium channels and slow Na channel inactivation and deactivation. Lqh1-induced channel block depended upon the holding voltage and the test voltage used to elicit the Na currents. Evidence from sucrose-gap and voltage-clamp experiments suggest that all three toxins act in a voltage dependent fashion to bind to Na channels and block them as well as slow channel inactivation and closing.

Tu-Pos128

PROBING THE INNER PORE PROPERTIES OF BTX-MODIFIED SQUID NA CHANNELS WITH INTERNAL BLOCKERS. *Joëlle Tanguy and J. Z. Yeh.* Dept of Pharmacology, Northwestern Univ., Chicago, IL, and MBL, Woods Hole, MA.

We have previously reported that, in squid axons, BTX irreversibly alters the gating properties of the voltage-sensitive Na channels, abolishing inactivation and shifting the activation voltage dependency towards the negative direction by about 50 mV, as seen in other preparations. In addition, we showed that external divalent cations block both the BTX-modified channels and the unmodified channels, suggesting that the external mouth of the channel is not markedly affected by BTX-modification. In this study, using neutral, mono-, bis-, and tri-valent organic cations, we probed the inner mouth of the BTX-modified Na channel in internally perfused and voltage-clamped squid axons, by comparing the blocking action of these agents in BTX-modified channels and in pronase-treated channels, after the complete removal of the fast inactivation. BTX-modification altered drastically the blocking action of all the internal blockers tested. (1) The blocking potency of neutral as well as charged blockers was greatly reduced. The Kd values (at 0 mV) were ten- to several hundred-fold larger depending on the blocking agent, increasing from 0.8 mM to 11 mM for benzocaine, and from 380 μM to 60 mM for gallamine for example. (2) The voltage dependence of the block was suppressed in the presence of QX-314, QX-222, SC-31828, and gallamine, and greatly reduced for 9-AA and pancuronium. (3) The use-dependent block of all cationic blockers tested, such as QX-314, QX-222 and SC-31828, was abolished. (4) BTX-modification altered the interaction between internal Na ions and the charged blockers tested. In pronase-treated channel, increasing internal Na ion concentration enhanced the blocking potency and voltage dependency of such blockers as propyl-bis-guanidine, octyl-bis-guanidine, and gallamine, whereas, in BTX-modified channels, high internal Na concentration attenuated their blocking potency and eliminated the voltage dependency of the block. Thus, the marked decrease in the affinity of the internal blockers for their binding site induced by BTX-modification does not result from the removal of the fast inactivation *per se*, but from a structural modification of the inner mouth of the channel induced by BTX binding. (Supported by NIH grant GM 24866).

Tu-Pos129

EFFICIENT EXPRESSION OF RAT BRAIN SODIUM CHANNEL TYPE IIA α SUBUNITS IN CHINESE HAMSTER OVARY CELLS

T. Scheuer, J. T.W. West, L. Maechler and W.A. Catterall, Department of Pharmacology, SJ-30, University of Washington, Seattle, WA 98195

Chinese hamster ovary (CHO)K1 cells have been transfected with an expression vector containing cDNA coding for the Rat brain type IIA sodium channel α subunit. The sodium channel coding sequence was inserted into the vector ZemRVSP6 at the BamH1 site under control of the mouse metallothionein (mMT1) promoter. This vector also contains a neomycin-resistance gene conferring G418 resistance. CHO K1 cells were transfected with this construct by calcium phosphate precipitation. G418-resistant clones were selected and tested for the expression of full length sodium channel mRNA (6.5 kB) by Northern blot analysis. Expression of the 6.5 kB mRNA was increased 3-fold by 50 μM $ZnCl_2$ indicating that expression is under control of the exogenous mMT1 promoter. Clonal cell lines expressing 6.5 kB mRNA were tested electrophysiologically for sodium channel expresssion. One such clone, C81-11, expresses sodium currents ranging from 1.5-20 nA, mean I=5.6 nA, n=13. Peak sodium current occurred near 0 mV. Boltzmann fits to inactivation curves gave mean values of $V_{1/2}$=-50.4 mV, k=5.4 mV, n=11. Inactivation of the sodium current was rapid (T=514 μsec@0 mV). Control CHO K1 cells express much smaller sodium currents in our solutions. The α scorpion toxin from *Leirurus quinquestriatus* binds specifically to these cells with K_D =5 nM. ^{3}H-Saxitoxin binding to cells yields a channel density that is consistent with the currents measured electrophysiologically. The efficient expression of rat brain type IIA α subunits makes C81-11 cells an optimal system for both electrophysiological and biochemical studies of this channel type.

Tu-Pos130

MODULATION OF RAT BRAIN TYPE IIA SODIUM CHANNELS BY ACTIVATORS OF PROTEIN KINASE C.

R. Numann, T. Scheuer, and W. A. Catterall, Department of Pharmacology, SJ-30, University of Washington, Seattle, WA 98195

Sodium channels in cultured rat brain neurons and Chinese hamster ovary (CHO) cells transfected with rat brain type IIA sodium channel α subunits (C81-11), show a similar pattern of modulation by whole cell activators of protein kinase C. Na channels from cultured rat brain neurons (embryonic day 20) were studied using whole cell voltage clamp with a pipette solution containing 1 mM ATP and 140 mM NaCl. Choline-Cl was substituted for external Na to prevent regenerative activity and outward sodium currents were recorded. When the phorbol ester phorbol 12-myristate 13-acetate (PMA) was applied at 25-250 nM, the peak Na current was quickly (< 3 min.) reduced by 15-50%. Four out of six cells showed a concurrent small slowing of the inactivating phase of the Na current. Continued recording in the presence of PMA showed no further reduction in peak current but macroscopic inactivation became 2-3 times slower than control. These effects could be seen at all test voltages. Identical effects were obtained by the lipid soluble diacylglycerols 1-Oleoyl-2-Acetyl-sn-Glycerol (OAG) and 1,2-Dioctanoyl-rac-Glycerol (DOG) at concentrations of 250-1000 nM.

C81-11 CHO cells transfected with cDNA encoding rat brain IIA α subunits express functional Na channels at high density. Cell-attached patches contain several channels and can be used to record both macroscopic and single channel currents. OAG at 500 nM produced a two- to five-fold suppression of the peak Na current and a slight slowing of inactivation. As in brain neurons, inactivation slowed progressively and, in two patches, the remaining Na channels were converted to a form with little or no fast inactivation. The effect of purified protein kinase C applied to the cytoplasmic surface of excised inside-out patches from C81-11 cells is under investigation. In two patches studied thus far, protein kinase C at 1.5 nM produced a 4-5 fold down regulation of the sodium current. Further experiments with excised patch recording are in progress.

Tu-Pos131

SITE-DIRECTED ANTIPEPTIDE ANTIBODY, AB_{SP31}, INHIBITS THE BINDING AND FUNCTIONAL EFFECTS OF α-SCORPION TOXIN.

Galen Eaholtz, Todd Scheuer and William A. Catterall, Graduate Program in Neurobiology and Department of Pharmacology, SJ-30, University of Washington, Seattle, WA 98195.

Alpha-scorpion toxin from Leiurus quinquestriatus (LqTx) slows sodium current inactivation when applied to primary fetal rat brain cell cultures and Chinese hamster ovary cells which are stably transfected with rat brain type IIA sodium channel α-subunit DNA. Site-directed antipeptide antibody AB_{SP31}, which recognizes the amino acid sequence of residues 355 to 371 of the rat brain sodium channel (RII; predicted from the cDNA of Noda et al., 1986, *Nature* 320:188-192), inhibits ^{125}I-LqTx binding to an extracellular site on domain I of sodium channels (Thomsen and Catterall, 1989, *PNAS* 86:10161-10165). We studied electrophysiological effects of AB_{SP31} and its interaction with LqTx in both primary cultured brain neurons and C81-11 cells (Scheuer et al., 1991, *Biophys. Abstr.*) using whole-cell voltage clamp (Hamill et al., 1981, *Pflügers Arch.* 391:85-100). Sodium currents were measured during a 12.5 msec test pulse to 0 mV from a holding potential of -80 mV preceded by a 100 msec prepulse to -120 mV. Our results from rat brain cells and C81-11 cells show: (1) sodium current activates and inactivates completely within 4.5 msec during the test pulse for both cell types; (2) 100 nM LqTx slows sodium current inactivation within 30 sec of application to both cell types; and (3) AB_{SP31} dramatically inhibits the development of the LqTx effect on sodium current inactivation. We conclude that AB_{SP31} blocks the binding and functional effects of LqTx on macroscopic sodium current inactivation. Studies of site-directed antibodies recognizing other regions of the sodium channel are in progress.

Tu-Pos132

EFFECTS OF SAXITOXIN, DIVALENT CATIONS AND LIDOCAINE ON TETRODOTOXIN-SENSITIVE AND TETRODOTOXIN-RESISTANT SODIUM CHANNELS IN RAT DORSAL ROOT GANGLION NEURONS.

M.-L. Roy and T. Narahashi, Dept. of Pharmacol., Northwestern Univ. Med. Sch., Chicago IL 60611.

Dorsal root ganglion neurons acutely dissociated from 3-10 day old rats express tetrodotoxin (TTX)-sensitive (TTX-S, K_d~1 nM) and TTX-resistant (TTX-R, K_d~100 μM) sodium channel currents in varying proportions. Currents were recorded using the whole cell patch clamp techniques. TTX-R current was more likely to be present in younger animals (3-7 days), whereas TTX-S current was more common in older animals (7-10 days), and these current types differed in activation and inactivation kinetics (Roy and Narahashi, Neuroscience Abstr. #84.4, 1990). These current types have been found to differ in their responses to other pharmacological agents. Saxitoxin (STX) at 10 μM abolished TTX-S current, but TTX-R current showed no significant reduction in peak current amplitude. Several external divalent cations exerted different effects on these current types. TTX-S current was reduced <50% by 50 μM Pb^{2+}, 5 mM Ca^{2+}, and 5 mM Cd^{2+}, with a maximum current-voltage (I-V) shift of +12 ± 3 mV occurring with Cd^{2+}. TTX-R current, however, was reduced <30% by 5 mM Ca^{2+} and <60% by 50 μM Pb^{2+} and 5 mM Cd^{2+}, with a +20 ± 3 mV I-V shift occurring with Pb^{2+} and Cd^{2+}. The actions of the local anesthetic lidocaine also showed differences between TTX-R and TTX-S currents. Dose-response curve data indicated that TTX-S current is slightly more sensitive to tonic block by 1-100 μM lidocaine than TTX-R current. However, TTX-R current is more susceptible than TTX-S current to use-dependent block by lidocaine at frequencies ranging from 1 to 33.3 Hz. The contrasting biophysical and pharmacological characteristics of TTX-S and TTX-R sodium channels are of importance in discerning the role of these channels in CNS development and drug action. Supported by NIH grants RO1 NS14144 and F31 MH09839.

Tu-Pos133

CHARACTERIZATION OF A QUATERNARY AMMONIUM BINDING SITE IN SINGLE BATRACHOTOXIN-ACTIVATED Na^+ CHANNELS.

G. K. Wang, R. Simon and S.-Y. Wang*
Dept. of Anesthesia, Harvard Med. Schl. & Brigham and Women's Hospital, Boston, MA and *Dept. of Biology, SUNY at Albany, NY.

A quaternary ammonium (QA) binding site was identified in single BTX-activated Na^+ channels derived from rabbit skeletal muscle and incorporated into planar bilayers. Alkyl trimethylammonium (alkyl-QA) derivatives from decyl-(C10) to octodecyl-(C18) QA compounds were tested. Under symmetrical 200 mM NaCl conditions, these QA compounds induced channel closures with mean closed times (τ_c) ranging from ≤10 ms to 500 ms at +50 mV. The structure-activity study showed that the longer the alkyl chain present, the longer the τ_c induced. Hence, the off-rate constants ($1/\tau_c$) were inversely related to the carbon number in the alkyl chain. In contrast, on-rate constants were maximal for the C14-QA compound but sharply declined for the C18-QA compound. The estimated $\Delta G°_{eq}$ of QA binding was ~450 calories per carbon atom with K_d =25 μM for C10-QA and about 0.25 μM for C16-QA. The binding of these QA compounds was strongly voltage-dependent; depolarization increased the binding affinity by about 8-10 fold per 100 mV. Furthermore, both the on- and off-rate constants of QA binding were affected by the concentration of Na^+ ions. The calculated K_d values were significantly lower at 50 mM NaCl than that at 500 mM. Similar Na^+ ion effects and voltage-dependent binding were found previously for cocaine-Na^+ channel interactions. In addition, cocaine and mepivacaine were found to compete directly with QA compounds for a single local anesthetic (LA) binding site. We conclude that (a) there is a QA binding site in BTX-activated Na^+ channels; (b) the environment of the QA binding site is relatively hydrophobic; (c) the QA dwell times are largely determined by the hydrophobic interactions with Na^+ channels; and (d) this QA binding site probably overlaps with the LA binding site. Supported by NIH GM35401.

Tu-Pos134

Perforated Patch Recordings with Amphotericin B

James Rae, Kim Cooper, Peter Gates, and Mitch Watsky
Depts. of Physiology and Biophysics and Ophthalmology
Mayo Foundation, Rochester, MN.

We have used Amphotericin B in a patch pipette filling solution to permeabilize membrane patches, an approach modified from Korn and Horn (J. Gen. Physiol. 94:1989) for Nystatin. With blunt pipettes pulled from KG-12 or 8161 glass and having 1-2 MΩ resistances, we achieve access resistances of 3-4 MΩ for whole cell recordings. With less ideal tip geometrics from several other glass types, we have achieved access resistances of <10 MΩ for 13 different cellular preparations. In addition to recording whole currents without loss of cellular macromolecules, these perforated patch approaches allow much better whole cell capacity compensation than routinely possible in our preparations by "standard" whole cell recording methods.

Perforation of the membrane in the pipette after patch excision and vesicle formation results in a recording situation analogous to an outside-out patch. Using this approach, we have been able to record for up to 30 minutes from inward rectifier channels in chick lens epithelium, channels which disappear within a few seconds in a standard inside-out patch configuration.

We explored the use of the "perforation" approach to measure resting voltages in epithelial monolayers in current clamp. We used a preparation which contained stretch-activated non-selective cation channels so that we could independently measure the resting potential just before the amphotericin partitioned. The two approaches agreed to within about 5 mV.

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Tu-Pos135

G PROTEIN ACTIVATORS INDUCE A NON-INACTIVATING OUTWARDLY RECTIFYING K CURRENT IN MURINE MACROPHAGE-LIKE J774.1 CELLS. L.C. McKinney and E.K. Gallin. Dept. of Physiology, Armed Forces Radiobiology Research Institute, Bethesda, MD. 20889.

Some agents (such as the tripeptide fMLP) that activate macrophages act via GTP-binding proteins, and also produce changes in membrane potential (Vm). The ionic basis of the Vm changes is not known, and activation of specific ionic conductances by G proteins has not been demonstrated in the macrophage. In this study, the whole-cell patch clamp technique was used to test whether intracellular perfusion of adherent J774.1 cells with compounds that cause activation of G proteins would affect ionic currents. Composition of the pipette solution was (in mM): 145 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, pH 7.3. Cells were bathed in Na Hanks. When GTP-g-S (10 uM), GppNHp (10 uM), or AlF_4^- (200 uM) was included in the pipette, an outward, non-inactivating current appeared <5 min after patch disruption that was never observed in the absence of G protein activators, and was not linked to the persistence or decay of the inwardly rectifying (K_i) current that is normally present in J774 cells. 100 or 1 uM GTP-g-S respectively decreased or increased the time to onset of the outward current. Current was activated by depolarizing voltage steps from near E_K(-70 to -80 mV) and was outwardly rectifying. The magnitude of the steady state conductance ranged from 1-7 nS for 440 ms steps to 0 mV. Concomitent with the appearance of the outward current, Vm (zero current potential) hyperpolarized toward E_K, and remained so even after washout of the inward K current, indicating K selectivity. In addition, replacement of >95% of Cl by isethionate in the pipette and bath solutions did not prevent induction of outward current by GTP-g-S. Pre-exposure of the cells for 2-5 hrs with pertussis toxin (PTX; 250 ng/ml) inhibited the induction of outward current by GTP-g-S (10 uM;N=3). We conclude that a PTX-sensitive G protein modulates the appearance of a novel K current in J774.1 cells. A similar current has been described in rat basophilic leukemia cells (McCloskey and Cahalan, 1990).

Tu-Pos136

POTASSIUM CHANNEL FROM CARDIAC SARCOPLASMIC RETICULUM STUDIED INTO PLANAR LIPID BILAYERS.
H. Chabot and E. Rousseau, Dept. Physiol. and Biophys., Fac. of Medicine, Univ. of Sherbrooke, CANADA, J1H 5N4.

Cardiac and skeletal sarcoplasmic reticulum microsomal fractions were isolated by differential and sucrose gradient centrifugations (Meissner and Henderson 1987, J. Biol. Chem. 262). Fractions enriched in SR membrane were caracterized by isotopic $^{45}Ca^{2+}$ flux measurements, gel electrophoresis and [^{3}H]-ryanodine binding. Vesicles were fused (10-50µg of proteins) into planar lipid bilayers made of PE, PS, PC (5:3:2; 25 mg/ml of decane), in presence of either KCl or K-gluconate buffers, containing mM $CaCl_2$ (pH 7.2). Single channel recording were generally obtained in asymmetric buffer system (100 mM trans/300 mM cis). The channel activity is characterised by an ohmic behavior and a slow gating mode. The average unitary conductance of the cardiac SR K^+ channel is 135 ± 15 pS, with the presence of a frequent and stable subconducting state, refered as O_1 by Hill & al. (Biophysical J. 55, 1989). The variations of the reversal potential with changing K^+ concentration confirm the selectivity of this pathway for monovalent cations. The open probability of this channel is slightly voltage dependant: P_o increased with depolarizing voltages. The gating mechanism of this large conducting K^+ was not Ca^{2+}-dependant. However, changes in cis free [Ca^{2+}], from mM to µM, induced a decreased in unitary conductance that might be related to variations of surface charge density in vicinity of the channel protein or to a direct effect of divalent cations on this structure. Under similar experimental conditions, the skeletal SR K^+ channel shares several properties with a cardiac SR K^+ channel: high conductance (170 pS) presence of a subconducting state, cations selectivity, slow gating mode; suggesting that these channels have the same physiological role in these two kinds of striated muscles. The regulation of these K^+ channels by intracellular compounds that might control ions movement across the SR membrane are currently under investigation.

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Tu-Pos137

A DELAYED RECTIFIER POTASSIUM CHANNEL IN ISOLATED AIRWAYS SMOOTH MUSCLE (ASM) CELLS.
J.P. Boyle, M. Tomasic and M.I. Kotlikoff.
Dept. of Animal Biology, School of Veterinary Medicine, Univ. of Penn., Philadelphia, PA

We have investigated the single channel events underlying the inactivating component of the whole cell K^+ current in ASM. Recordings were made from on-cell and inside-out patches in acutely isolated ASM cells from porcine or canine trachealis. In addition to the previously described large conductance, Ca^{2+}-sensitive K^+ channel, we observed a low conductance (G=14.7±2.1pS) K^+-selective channel that was not sensitive to reductions in bath and/or pipette solution [Ca^{2+}] with EGTA. This channel was identified as a delayed rectifier (K^+_{dr}) by its slow activation (30ms to peak in ensemble current) and its time- and voltage-dependent inactivation. The time-dependent inactivation of the ensemble average current occurred over 2s, which is in good agreement with previously described whole-cell currents in ASM cells. Under similar conditions, no time-dependent inactivation of K_{Ca} was observed. Voltage-dependent activation or inactivation curves could be fitted to Boltzmann equations; V_{50}=5.5mV for activation and V_{50}=-53mV for inactivation, the slope factors were K_{act}=6 and K_{inact}=9.8. Combining the activation and inactivation curves of this channel does not produce a voltage window in which K^+_{dr} would show a significant P_{open} suggesting that this channel opens transiently in response to depolarization. K^+_{dr} was not sensitive to TEA (0.5-2mM) or charybdotoxin (100nM) but was inhibited by 4-aminopyridine (1mM), which is in good agreement with data obtained from whole-cell recordings. These data suggest that K^+_{dr} is responsible for the inactivating K^+ current in ASM cells.

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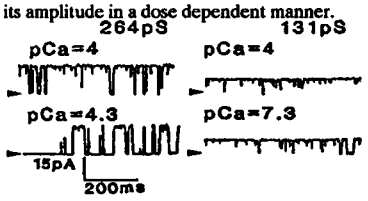
Tu-Pos138

POTASSIUM CHANNELS FROM HUMAN MYOMETRIUM INCORPORATED INTO LIPID BILAYERS. Pérez G., *Ludmir J., *Erulkar S. D., Toro L., and Stefani E. Dept. Molecular Physiol. & Biophys. Baylor College of Medicine, Houston, TX 77030, and *Dept. Pharmacology, Univ. Pennsylvania, Philadelphia, PA 19104.

Plasma membrane vesicles from healthy human myometrium were incorporated into planar lipid bilayers to record K channel activity. We have identified two types of K channels, a large conductance (264±3 pS, n=8) calcium activated K (K_{Ca}) channel and a calcium insensitive K channel of smaller conductance (146±10 pS, n=5). The small conductance channel had similar open probability (Po) at pCa 4 than at pCa 7.3 (see figure). This channel seemed to be ATP sensitive, since in one experiment 1 mM ATP inhibited channel activity. Since the K_{Ca} channel was more frequently observed this study was focused to determine its characteristics. The Po vs. voltage relationship and the internal calcium concentration ($[Ca^{2+}]_i$) dependence of the K_{Ca} channel were analyzed. Po vs. voltage curves were fitted to a Boltzmann distribution at different $[Ca^{2+}]_i$. Their half activation potential was shifted from -66.5 mV (pCa=4.6) to +113 mV (pCa=6.3) without a significant change in the slope factor (k=15 mV). The affinity of the channel for Ca^{2+} was calculated at several voltages. At -60 mV the half activation concentration was $K_{1/2}$=31.7±11.2 μM, (n=3) and at -40 mV $K_{1/2}$ was 22.6±10.6 μM, (n=3). Neither apamin (up to 100 nM, n=3) nor 4-aminopyridine (up to 1 mM, n=2) showed any effect either when added to the outside or to the inside of the channel. On the other hand, external charybdotoxin (20 nM) completely blocked channel activity recorded at -40 mV. However, the activity could be restored after pulsing the channel to positive potentials. No changes in amplitude were observed. Addition of TEA to the external side of the channel reduced its amplitude in a dose dependent manner. This effect was more prominent at negative potentials. Kd was 281 μM at +40 mV and 187 μM at -40 mV. K channels from human myometrium may be studied with the bilayer technique in an attempt to elucidate their role in uterine contraction. In the figure arrows mark the closed state. Supported by NIH grants HD-25616 (E.S.) and NS-12211 (S.D.).



Tu-Pos139

TWO OUTWARD POTASSIUM CURRENTS ON EMBRYONIC XENOPUS MUSCLE IN EARLY CULTURES. Frances Moody-Corbett, Division of Basic Sciences, Faculty of Medicine, Memorial University of Newfoundland, St. John's, NF.

Very little information is available on the developmental appearance of outward potassium currents on vertebrate skeletal muscle. The purpose of the present study was to examine these currents on embryonic frog muscle at early times in culture. Myotomal muscle cells removed from 1 day old Xenopus embryos were put in culture and macroscopic currents were recorded using the whole cell patch clamp technique beginning 2-3 hr after plating. The muscle cells were held near resting membrane potential (-70 mV) and the current recorded during 100 msec step depolarizations. The extracellular solution contained (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES and 1 μg/ml tetrodotoxin and the patch electrode contained (in mM) 140 KCl, 1 EDTA or BAPTA, 5 $MgCl_2$ and 10 HEPES. Outward potassium currents, which were activated at potentials more positive than -30 mV, first became apparent after 4-5 hr in culture and within the first 12 hr most muscle cells showed one of two types of outward current. One group of cells (13/19) had an outward current that did not inactivate during a 100 msec step depolarization while the other group (6/19) showed an outward current that reached a peak amplitude within 10 msec and then inactivated with a single exponential decay rate. By 1 day in culture outward current became apparent at slightly more negative potentials (-40 mV) and the majority of cells had an outward current which inactivated and was best fit by a double exponential function. At 1 day in culture only a few cells showed no inactivation (2/73). The results suggest that the non-inactivating outward current may represent the earliest outward potassium current on these muscle cells and that by 1 day in culture the outward current is composed of an inactivating and non-inactivating component.

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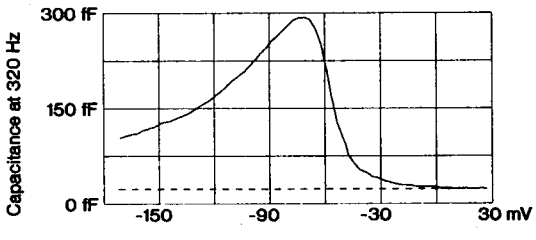
Tu-Pos140

STATE-DEPENDENT ELECTRICAL CAPACITANCE OF POTASSIUM CHANNELS

Stefan H. Heinemann and Walter Stühmer

Max-Planck-Institut für biophysikalische Chemie, Abteilung Membranbiophysik, D-3400 Göttingen, FRG.

Gating currents from various potassium channel species expressed in *Xenopus* oocytes were recorded from inside-out patches. Standard P/n procedures for capacitive transient cancellation could not be applied, because gating charge displacement already starts at potentials as negative as -160 mV. Therefore the passive electrical properties of the channel proteins were measured as a function of membrane voltage employing high-resolution phase-sensitive Lock-In techniques. The determined voltage-dependent capacitance as shown in the figure gives insight into the rather complex gating mechanism even in a voltage range where the measurement of transient gating currents is not feasible. Models assuming four independent gating particles plus charge immobilization for open channels were sufficient to describe the observations at moderate depolarizations. In particular at high stimulating frequencies a residual capacitive component was found at negative potentials indicating that the dielectric constant of the channel protein increases upon channel closing.



Tu-Pos141

PASSIVE ELECTRICAL PROPERTIES AND WHOLE-CELL IONIC CURRENTS RECORDED IN FRESHLY ISOLATED RABBIT CORONARY ARTERY SMOOTH MUSCLE CELLS. WAN, Xiaodong, and Normand LEBLANC. Div. of Cardiovascular Sci., St. Boniface Gen. Hosp. Res. Centre, Dept. of Physiol., University of Manitoba, Winnipeg, Manitoba, CANADA R2H 2A6.

Single smooth muscle cells from the rabbit left descending coronary artery were isolated using collagenase and protease. The whole-cell configuration of the patch clamp technique was used to measure the cell electrical constants and macroscopic ionic currents. These cells were spindle-shaped and relaxed when superfused with normal Hepes buffered solution containing 1.8 mM Ca^{2+}. With an internal solution containing low EGTA (0.1 mM), 140 mM K^+ and 5 mM ATP, the cells exhibited a mean resting membrane potential of -53 ± 5.2 mV (n=8). From current clamp experiments the input resistance R_{inp} = 670 ± 234 MΩ (n=4) and membrane capacitance C_m = 22.0 ± 0.53 pF (n=9). Under voltage clamp condition, from a holding potential of -60 mV, time independent (I_{bckg}) and time-dependent (I_t) outward currents could be elicited. Interestingly I_{bckg} was found to reverse very near the RMP measured in the same cell. I_t activated near -30 mV and increased non-linearly with membrane depolarization as expected from outwardly rectifying membrane properties. Long test pulses revealed that I_t inactivated slowly with time. Consistent with the idea that a component of I_t inactivates in a time- and voltage-dependent manner, holding the membrane potential more negative (-80 mV) enhanced the size of I_t. Exposure of the cells to 5.4 mM $[Ca^{2+}]_o$ enhanced I_{bckg} and I_t, shifted the activation of I_t towards negative potentials and hyperpolarized the RMP. Conversely, dialyzing the cells with 5 mM EGTA, or superfusion with a nominally Ca^{2+}-free external solution had the opposite effects. These experiments likely reflect the activity of Ca^{2+}-dependent K^+ channels as shown in many other smooth muscle cell preparations. They also stress their importance in generating a well polarized resting membrane potential in these cells. Supported by the Manitoba Heart Foundation. N.L. is a Scholar of the MRC of Canada.

Tu-Pos142

EVIDENCE FOR A DELAYED RECTIFIER POTASSIUM CURRENT, I_K, IN RABBIT ATRIAL MYOCYTES. B. Fermini, S. Nattel. Research Center, Montreal Heart Institute, Montreal, Quebec, Canada H1T 1C8.

The transient outward current, Ito, is believed to play a major role in determining action potential duration (APD) in rabbit atrium yet in view of its slow reactivation kinetics, this current may not contribute to atrial repolarization under normal sinus rhythm. Thus other K^+ currents may contribute to the repolarization phase of this cardiac tissue. Using the patch-clamp technique, the delayed rectifier potassium current (I_K) was identified and its properties were studied in myocytes isolated from rabbit atrium (HEPES buffered Tyrode with Co^{2+} to block the Ca^{2+} current, T= 30°C). The pipette solution contained (in mM): KCl 130; $MgCl_2$ 1.0; MgATP 5.0; EGTA 5.0; HEPES 10.0, pH adjusted to 7.3 with KOH. Depolarizing voltage steps applied from a holding potential (HP) of -40 mV elicited a time- and voltage-dependent outward current (I_K) which activated with a sigmoïdal time course, as well as a tail current (I_{Kt}) apparent upon return to HP. No inactivation of the current was apparent for depolarizing pulses of up to 2.5 sec. Both components could be increased by raising the bath temperature (to 35 °C) or addition of isoproterenol (10μM) to the superfusate solution. The reversal potential of the tail current was -77.3 ± 1.3 mV (n=6, $[K^+]_o$ 5.4 mM), which is about 10 mV less negative than E_K assuming $[K^+]_i$ to be 140 mM, indicating that the contribution of other ions to this current cannot be neglected. The half-activation potential obtained by averaging normalized tail amplitude from 7 cells was -4.0 mV and the Boltzmann equation slope factor (k) was 12.3 mV. The threshold voltage for the activation of I_K was approximately -40 mV and saturation occurred near +50 mV. The time-dependency of I_K conformed to the "envelope test" of tail currents when the membrane was depolarized to +60 mV from a holding potential of -40 mV. In conclusion, a K^+ current component showing properties similar to the ones described for I_K in ventricular preparations is present in rabbit atrial myocytes, and may play a yet unsuspected role in determining APD in this structure, especially under conditions when the heart rate is increased.

Tu-Pos143

CAPSAICIN BUT NOT RESINIFERATOXIN INHIBITS K^+ CURRENTS IN ISOLATED RAT VENTRICULAR MYOCYTES. Neil A. Castle. Anesthesia Research Laboratories, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

Capsaicin (CAPS) is a pungent irritant found in red peppers. Its major target of action is believed to be substance P-containing sensory neurons. CAPS also prolongs the action potential (AP) in atrial muscle of heart, an action which has been proposed to result from the release of calcitonin gene related peptide, which in turn enhances inward Ca^{2+} currents (I_{Ca}). However, in neurons CAPS has been shown to inhibit K^+ currents. Such an action could possibly contribute to a CAPS-induced prolongation of the AP. To investigate this possibility I examined the effects of CAPS on the transient outward K^+ current (I_{to}) and the inward rectifier K^+ current (I_{K1}) in adult rat ventricular myocytes, using the whole cell variant of the patch-clamp technique in the presence of 10 μM tetrodotoxin and 3 mM Co^{2+} to block I_{Na} and I_{Ca}, respectively. CAPS reduced the peak amplitude of I_{to} and increased the rate of current inactivation in a dose-dependent manner, exhibiting an IC_{50} of 6.4 ± 0.6 μM (n=5). The inactivation of I_{to} in the absence of CAPS was well fit by a single exponential (77 ± 2 ms, n=8 at +40 mV). However, in the presence of 10 μM CAPS inactivation was best fit by two exponentials, τ_{fast} 3.9 ± 0.4 ms; τ_{slow} 92.0 ± 3.0 ms (n=8 at +40 mV) with the fast component constituting 46% of the total current. CAPS (10 μM) only induced a small hyperpolarizing shift (4 mV) in the voltage-dependence of steady-state inactivation which was not statistically significant. Similarly, CAPS (10 μM) had no effect on the rate of recovery from inactivation, τ = 49 ms and 48 ms for control and drug, respectively. No cumulative (or use-dependent) inhibition was observed during repetitive depolarizations to +40 mV (1 Hz). The CAPS homolog, resiniferatoxin, which as an irritant is up to 10^4 times more potent than CAPS, had no effect on I_{to} up to concentrations of 10 μM. CAPS also inhibited I_{K1}. However, its potency was ~ 8-fold less than for inhibition of I_{to}, the IC_{50} being 47 ± 4 μM (n=6). Since I_{to} is a major contributor to atrial muscle repolarization, inhibition of this current by CAPS may be involved in its AP prolonging action in this tissue. Supported by an AHA postdoctoral fellowship to NAC and GM 15904 to GR Strichartz.

Tu-Pos144

VOLTAGE ACTIVATED K^+ CHANNELS IN ISOLATED CILIATED CELLS FROM AIRWAY EPITHELIA. J.I. Kourie, B. Ribalet, A.B. Lansley, M.J. Sanderson, and E.R. Dirksen. Dept. of Anatomy and Cell Biology, UCLA Medical Center, Los Angeles, CA 90024.

The electrical properties of the basolateral membrane of enzymatically-isolated ciliated cells were studied by application of the patch-clamp technique. In the whole-cell configuration, depolarizing voltage-clamp steps induce outward membrane currents which can be dissected into: (a) An instantaneous time-independent leak current (I_l), and (b) a time- and voltage- dependent current which at the steady-state is referred to as I_{ss}. I_{ss} consists of I_l and an outward rectifying K current (I_K=I_{ss}-I_l). I_K is voltage-dependent, eliminated by substituting the K^+ in the pipette solution for Na^+ or Cs^+ and blocked with 10mM TEA-Cl in the bath solution or 10mM Cs^+ in the pipette solution. At a membrane voltage (V_m) of 140mV, the contributions of the potassium conductance, (G_K= I_K/V_m-E_K) and the leak conductance (g_l), to the conductance of the basolateral membrane were 10.98nS and 10.32nS, respectively. The time course of the activation of I_K is described with two exponentials. The time constants τ_1 and τ_2 were both voltage dependent, increasing from τ_1 = 6ms and τ_2 = 81ms, at 30mV, to τ_1 =58ms and τ_2 = 370ms at 100mv, and then decreasing to τ_1 = 16ms and τ_2 = 147ms at 170mV. Corresponding single-channel activities were observed in the intact membrane and in excised patches. In the cell-attached configuration the K^+ channel has a conductance of 85.7pS at a V_m of 140mV. A typical isolated cell has a diameter of 15μm and 75% of its surface area is basolateral membrane. Hence, at a V_m of 140mV, the comparison of whole-cell K conductance (G_K) with that of a single K^+ channel (g_K) indicates that an isolated ciliated cell has an average of 128 such channels, yielding a density of 0.24 channel/μm^2. Activation of these K^+ channels at physiological voltages may play a role in re-establishing the electrochemical gradient required for the activation of Cl^- channels in the apical membrane. Supported by the NIH and Cystic Fibrosis Foundation.

Tu-Pos145

ION CHANNEL PROPERTIES ARE ALTERED BY LIPID BILAYER COMPOSITION. H. M. Chang+, S. M. Gruner* & R. Gruener+. +Dept. of Physiology, University of Arizona and *Dept. of Physics, Princeton University.

To gain a better understanding of the effects of the lipid environment on ion channel behavior, we examined the properties of the calcium-activated potassium (CaK) channel, from rat brain, reconstituted into lipid bilayers of different composition. Channel conductance and kinetic properties were similar when incorporated into phosphatidylethanolamine / phosphatidylserine (PE/PS) bilayers with a weight ratio varying from 30/70 to 90/10. In bilayers made from pure lipids [1-palmytoyl-2-oleoyl phosphatidylethanolamine (POPE), di-oleoyl phosphatidylcholine (DOPC) and monomethylated-dioleoyl phosphadityle-thanolamine (Me-DOPE)], the CaK channel conductance was similar at 233 and 210 pS (DOPC and Me-DOPE membranes) and 243 pS (POPE membranes). This compares to a conductance of 310 pS in PE/PS (55/45) reference membranes (linear slope conductances were measured between +10 and -27 mV). The mean opentimes were reduced from 12.0 (PE/PS bilayer) to 3.0, 1.6 and 1.3 msec (DOPC, Me-DOPE and POPE, respectively). The probability of the channel being open (as a percentage of total data collection time) was reduced from 61 (PE/PS) to 0.5, 1.2 and 3% (DOPC, Me-DOPE and POPE). Surface charge, spontaneous curvature or lipid-protein affinity may be responsible for the modifications of channel properties. These results suggest that channel properties during development, aging and consequent to exposure to modulating drugs may be significantly affected by the lipid composition of the cell membrane into which channels are incorporated.

Supported by an institutional BRSG grant (U. of Arizona).

Tu-Pos146

Activation of Muscarinic K^+ Channels following Parasympathetic Innervation in Embryonic Chick Heart: Relationship to the G Proteins L.Sen, J.V. Barnett & J.B. Galper. Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115

The mechanism by which parasympathetic innervation in developing heart induces the functional expression and coupling of muscarinic acetylcholine receptors (mAChR) to an inwardly rectifying K^+ channel is poorly understood. The purpose of this study has been to determine whether the parasympathetic innervation of embryonic heart increases the expression of mAChR-coupled K^+ channels (K_{ACh} channels) per se, and/or increase the avialability of G proteins which couple the muscarinic receptor to the inward rectified K^+ channel. The K_{ACh} channel has been examined using whole cell patch clamp technique. Embryonic chick (3-4 days *ovo*) myocytes with or without co-cultured ciliary ganglia were used. When cells were dialysed with either 100 μM GTP or 100 μM GTPγs (present in pipette), ACh-induced K^+ currents in control and co-cultured myocytes were similar (121 ± 23 and 128 ± 21 pA, n=15, p>0.05). However, in the absence of GTP or GTPγs in the pipette, ACh-induced K^+ currents were 3.2 fold greater in co-cultured cells than in controls (14±5 pA and 45±7 pA, respectively, n=18, p<0.01). The ACh activated currents in both control and co-cultured cells were completely abolished following pretreatment (10 hours) with pertussis toxin (n=5, p>0.05). Interestingly, the G protein coupled enhancement of Ca^{++} currents by isoproteronol was found to be similar in both control and co-cultured cells whether GTP or GTPγs was absent or present in the pipette. These results suggest that parasympathetic innervation of developing heart increases the expression of K_{ACh} due to the enhanced availability of the pertussis toxin sensitive G protein.

Tu-Pos147

UNUSUAL PROPERTIES OF OUTWARD CURRENTS IN LOBSTER STOMATOGASTRIC NEURONS

B.R. Jones and D.K. Hartline. Békésy Laboratory of Neurobiology, University of Hawaii, Honolulu, Hawaii 96822

Ionic currents were measured in somata of neurons from the lobster (*Panulirus*) stomatogastric ganglion using two-microelectrode voltage clamp at 22° C. Two large outward currents are observed in these cells with depolarizing pulses: a fast, transient current (I_A, 4AP sensitive), and a Ca^{++}-dependent, transient current (I_J, TEA and Cd^{++} sensitive).

Neither of these currents exhibits the dependence on $[K^+]_o$ expected from the Nernst equation. Slopes obtained from plots of $e^{E_{rev}F/RT}$ *vs* $[K^+]_o$ for the tail reversals of these currents yield ~ 500 mM for $[K^+]_i$, suggesting that the cells are loading with K^+ from the microelectrodes. In addition, the non-zero intercept of these plots suggests that either a second ion contributes to each of the two currents (P_X / P_K ~ 0.07), or $[K^+]_o$ near the membrane is ~ 30 mM higher than that in the bulk solution. We have found no evidence for a second ion that contributes to I_J.

The amplitude of I_A obtained by current subtraction is less than the value obtained in the same cell after blocking I_J with Cd^{++} or TEA. This apparent coupling between two distinguishable ionic currents may be explained by an extracellular resistance in series with the channels. Series resistance can be calculated from I(V) curves obtained before and after blocking I_J.

Our model of these somata involves a diffusionally restricted extracellular space that contributes a series resistance shared by I_A and I_J, and is capable of trapping extracellular K^+. This space might be associated with membrane infoldings or glial cells, both of which have been observed in this preparation.

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Tu-Pos148

SINGLE CHANNEL ANALYSIS OF THE BLOCK OF TRANSIENT K^+ CURRENT(I_{to}) BY TEDISAMIL IN RAT VENTRICULAR MYOCYTES.

H.D. Lux, I.D. Dukes, & M. Morad., Dept. of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085 & M.D.I.B.L., Salsbury Cove, ME 04672.

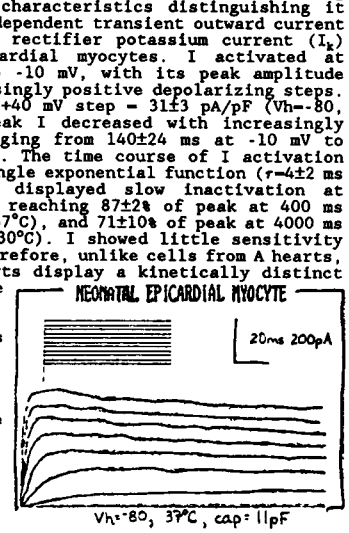
Tedisamil is a novel blocker of transient and delayed K^+ channels (I_{to}, I_K, I_A) in cardiac and glial cells (Dukes et al., 1990, J.P.E.T.: 254, 560-9). 10 μM tedisamil suppressed both I_A and I_{to} by enhancing the inactivation of I_{to} without a significant effect on peak current, consistent with a rapid open channel block scheme. In order to examine the mechanism of action of tedisamil further, unitary currents were recorded from cell-attached membrane patches in intact ventricular myocytes. The data was sampled at 10 KHz and was filtered at 1.8 KHz. The pipette solution contained (in mM) 120 KCl, 4 NaCl, 5 Mg-ATP, 14 EGTA, 10 Hepes, pH 7.2. The experimental cell was bathed in a similar solution without EGTA and ATP. Unitary currents were activated by depolarizing pulses from -80 mV to +60 mV (inside) to examine I_{to} in the presence and absence of tedisamil. Unitary conductance for I_{to} channel was 15.7 pS in symetrical K^+. The mean open times were best fit with one exponential with a time constant of 1.50 ms in control and 0.84 ms in 50 μM tedisamil. The mean closed times were best fit with two exponentials. Tedisamil prolonged the mean closed time from 3.8 to 7.9 ms. The most prominent effect of tedisamil was to prolong the interval between the bursts. The peak average ensemble current was also reduced in 50 μM tedisamil and its rate of inactivation was markedly enhanced. Thus, tedisamil block of I_{to} was accompanied by shortening of mean open times and prolongation of intervals between the bursts. The data suggests that in addition to open channel block, tedisamil at 50 μM concentrations may also interact with the closed state of the channel. (Supported by NIH grant HL 16152.)

Tu-Pos149

A UNIQUE OUTWARD CURRENT IN NEONATAL CARDIAC MYOCYTES

Cynthia D. Jeck, Lisa Ebihara, Penelope A. Boyden
Columbia Univ., Dept. of Pharmacology, NY, NY 10032

Action potentials from adult (A) and neonatal (N) canine ventricular epicardial tissue display different time courses of repolarization. To understand the currents responsible for these differences we used the whole cell voltage clamp technique to study cells freshly isolated from N (<2 weeks old) left ventricular epicardium. Cells were studied under experimental conditions in which currents other than potassium currents were largely suppressed (2 mM Mn^{++}, 15-30 uM TTX, 30° or 37°C). In response to depolarizing clamp steps (Vh=-80 or -60 mV) 23% (17 out of 75) of cells displayed a slowly activating current (I) that had characteristics distinguishing it from both the voltage-dependent transient outward current (I_{tol}) and the delayed rectifier potassium current (I_k) recorded from A epicardial myocytes. I activated at potentials positive to -10 mV, with its peak amplitude increasing with increasingly positive depolarizing steps. The average peak I at +40 mV step = 31±3 pA/pF (Vh=-80, n=10). The time to peak I decreased with increasingly depolarized steps, ranging from 140±24 ms at -10 mV to 15±2 ms at +50 mV 37°C. The time course of I activation was best fit using a single exponential function (τ=4±2 ms at +40 mV, n=10). I displayed slow inactivation at depolarized potentials reaching 87±2% of peak at 400 ms after depolarization (37°C), and 71±10% of peak at 4000 ms after depolarization (30°C). I showed little sensitivity to 4-aminopyridine. Therefore, unlike cells from A hearts, some cells from N hearts display a kinetically distinct outward I and no A type I_{tol} or I_k, suggesting that expression of outward currents varies with development. However, because this I is present in only 23% of the N cells, it is unclear what role the current plays in N canine epicardial repolarization. (Supported by NHLBI HL28958).



Tu-Pos150

EFFECTS OF INTRACELLULAR Na ON MEMBRANE K CURRENTS IN MAMMALIAN VENTRICULAR MYOCYTES. N Chandramani, G-N Tseng. Pharmacology, Columbia U. New York

A number of abnormal conditions induce an elevation in $[Na]_i$. $[Na]_i$ has been shown to decrease or increase various membrane K currents. We examined the effects of $[Na]_i$ on membrane K channels in dog and guinea pig ventricular cells. $[Na]_i$ was altered by dialyzing the cell with pipette solutions containing different [Na] using the pipette perfusing method. Choline Cl was used as an substituent for NaCl to maintain the internal osmolarity. Experimental conditions were designed to eliminate current changes due to membrane transport systems activated by Na_i: external solution contained ouabain 20 uM, and both internal and external solutions were Ca-free with pH buffered by HEPES. Raising $[Na]_i$ from 0 to 50 mM caused an inward shift in membrane current at voltages negative to -90 and -55 mV (E_{thr}) at 2 mM K_o and 4 mM K_o, respectively (n=4 each), but caused little or no change in current in the voltage range between E_{thr} and +20 mV. The increase in inward current at negative voltages was abolished by 1 mM Ba, but insensitive to 4 mM 4-AP or 20 mM TEA, suggesting that Na_i may affect the inward rectifier channel (I_{K1}). Raising $[Na]_i$ increased the calculated maximum conductance of I_{K1} by an average of 25 % and 13% at 2 mM K_o and 4 mM K_o respectively (n=3 each). In 3 out of 9 cells, elevating $[Na]_i$ induced an outward current >100 pA at voltages positive to +30 mV. In conclusion, under our experimental conditions, the main effect of elevating $[Na]_i$ was an increase in inward current through I_{K1} channels at negative voltages .

Tu-Pos151

CHEMICAL GROUPS NEAR THE DIVALENT CATION BINDING SITE ON K CHANNELS. Ted Begenisich and Sherrill Spires. Department of Physiology, University of Rochester, Rochester, New York 14642.

External divalent cations produce significant alteration in the kinetics of delayed rectifier K channels of squid axons. The time constant for channel activation is slowed and the rate of deactivation is increased. These effects are produced by the physiologically important cation, Ca, but it is convenient to use Zn ions as probes for these studies (Gilly and Armstrong, J. Gen. Physiol. 79:965, 1982). The modifications produced by low concentrations (2 to 5 mM) of Zn ions are qualitatively similar to but much larger than those produced by much larger concentrations of Ca ions. At normal pH values (7.5), these Zn concentrations produce a 3- to 4-fold slowing of the activation time constant: much too large to be consistent with any reasonable voltage shift produced by interaction with surface charges. Zn ions interact with high affinity with histidine which led Gilly and Armstrong to speculate that the K channel divalent cation binding site might include histidyl residues. Furthermore, we have recently shown that histidine modifying reagents produce changes in K channel kinetics similar to those induced by Zn ions (J. Gen. Physiol. 96:*In Press*, 1990). However, we found that treatment of axons or giant fiber lobe cells with histidine reagents does not alter the actions of Zn, suggesting the binding site does not include important histidyl residues. Furthermore, from whole-cell patch clamp experiments on squid giant fiber lobe cells the pK for hydrogen ion inhibition of the Zn effects on channel kinetics is near 7.4, rather far from the "usual" pK of about 6.5 for histidine. Treatment with sulfhydryl reagents was also without effect on Zn action. In contrast, Zn ions had almost no effect on K channel kinetics after treatment with the amino-specific reagent trinitrobenzenesulfonic acid (TNBS). Consequently, the external divalent cation binding site on the squid delayed rectifier K channel probably contains one or more important amino residues: lysine or a terminal amine.

Tu-Pos152

MULTIPLE AMINOPYRIDINE BINDING SITES IN NEUROBLASTOMA K CHANNELS. J.K. Hirsh and F.N. Quandt. Multiple Sclerosis Center and Dept. of Physiology, Rush University, Chicago, IL 60612.

We have been studying the action of aminopyridine derivatives on voltage dependent K channels in N1E-115 neuroblastoma cells (NBC) grown in tissue culture using whole cell and excised membrane patch clamp techniques. Classically, in other excitable cells aminopyridines have produced block relieved by depolarization in a frequency-dependent manner. 4-Aminopyridine methiodide (4-APMI), is a membrane-impermeable derivative which blocks squid giant axon (SGA) K channels only from the cytoplasmic face (Kirsch and Narahashi (1983) J. Pharmacol. Exp. Ther. 226, 174-179). 4-APMI produced half-maximal block of the steady state current in neuroblastoma cells at a concentration of 2.1 mM in the extracellular solution as measured with whole cell voltage clamp. Block by 4-APMI was not relieved during a long depolarization (600 ms) nor was the block decreased when depolarizations were applied repetitively at intervals between 1000 to 60 ms. These results are similar to those obtained with 4-aminopyridine (4-AP) and 3,4-diaminopyridine (3,4- DAP), which however are both about an order of magnitude more potent in NBC at blocking the steady state whole cell current when applied extracellulary (half-maximal block at 0.3 and 0.5 mM, respectively). The equivalent potencies of 4-AP and 3,4-DAP in NBC also differs from SGA where 3,4-DAP is 50 times more potent (Kirsch and Narahashi, (1978) Biophys. J. 22, 507-512). Single channel analysis suggests that 4-APMI block differs from that produced by 4-AP. Ensemble currents obtained in the cell attached mode were reduced by extracellular 4-AP but were not reduced by extracellular 4-APMI. Thus 4-APMI cannot permeate neuroblastoma cells. In outside-out patches, 4-APMI (1 mM) reduced the open channel conductance by 25% but did not do so when applied to inside-out patches. The results of these studies suggest that neuroblastoma K channels differ in structure from SGA and other K channels. There may be binding sites for aminopyridines accessible from both the intracellular and extracellular surfaces. This multiplicity of binding sites may explain the lack of depolarization-induced unblock in the presence of these aminopyridine derivatives. Supported by the National Multiple Sclerosis Society.

Tu-Pos153

VOLTAGE-DEPENDENT BLOCK OF NEURONAL FAST CHLORIDE CHANNELS BY TETRAETHYLAMMONIUM ION. A. L. Blatz, D. Y. Sanchez, and M. L. Sigler. Department of Physiology, University of Texas Southwestern Medical Center, Dallas, TX 75235.

Tetraethylammonium ion (TEA) has been used extensively to block ionic currents through certain types of potassium-selective ion channels. We find that externally applied TEA, in the millimolar range, also blocks chloride-selective channels in acutely dissociated rat cerebral cortex neurons.

Excised, inside-out patches from surface membranes of cortex neurons usually contain 1-10 fast chloride channels with properties similar to those found in tissue-cultured rat skeletal muscle. Patch clamp techniques were used to examine the block of these channels by externally applied TEA. Extracellular (pipette) solutions contained (mM): 140 KCl, 5 TES, 1 EGTA, pH 7.0, and 0, 1, 5, 10, or 50 mM TEA (bromide or chloride salt). Intracellular solution contained (mM): 1000 KCl, 5 TES, 1 EGTA, pH 7.0.

In the absence of external TEA, the current-voltage relationship of neuronal fast chloride channel currents is relatively linear between -100 and +60 mV with a single channel conductance of about 140 pS. When the extracellular solution contained 1-5 mM TEA, single channel currents at membrane potentials more positive than the reversal potential (+25 mV) were relatively unaffected. Currents at potentials more negative than the reversal potential were blocked in a dose-dependent manner with the single channel conductance being reduced to 70 pS by 5 mM TEA. Raising external [TEA] to 10 mM caused only a slight additional reduction in chloride channel currents. TEA-blocked currents exhibited a linear current-voltage relationship at potentials more negative than the reversal potential. External TEA blocks with rapid kinetics, as the block was manifested as a reduction in single channel current amplitude with little effect on mean open or closed durations. Our results demonstrate a voltage-dependent block of neuronal fast chloride channels by TEA. Thus, caution should be exercised in the interpretation of experiments where TEA is used to implicate potassium channel involvement. Supported by NIH grant GM39731.

Tu-Pos154

INCREASED OPENING PROBABILITY (P_o) OF POTASSIUM CHANNELS IN THE ARTERIAL MUSCLE MEMBRANE OF GENETICALLY HYPERTENSIVE RATS. Sarah K. England, Theresa A. Wooldridge, Roberto G. De Lucena, William J. Stekiel, and Nancy J. Rusch. Dept. of Physiology, Med. Coll. of Wisconsin, Milwaukee, WI 53226.

Arterial muscle from genetic, renal and corticosteroid models of hypertension show an enhanced membrane permeability to potassium ions (K^+). To investigate the cellular mechanism for this, the biophysical properties of single K^+ channels were compared between arterial muscle from spontaneously hypertensive (SHR) and normotensive Wistar Kyoto (WKY) rats. In cell-attached patches exposed to pipette solution containing 145 mM KCl, both WKY (n=11) and SHR (n=12) aortic membranes consistently contained a 100 pS K^+-selective channel. The opening of this channel was voltage- and calcium-dependent, as evidenced by increased opening probabilities (P_o) measured during patch depolarization and the application of the calcium ionophore A23187 (10 μM). However, a 4-fold increase in the P_o of this 100 pS channel was seen in SHR aortic patches (SHR P_o = .23 ± .13; WKY P_o = .05 ± .02) at resting membrane potential (pipette potential = 0), reflecting an enhanced mean open time and increased number of events in the SHR arterial membrane. To determine if this enhanced P_o of K^+ channels in the SHR membrane was important in modulating arterial excitability, studies comparing the contractile responses of WKY and SHR aortic segments to K^+ channel blockers were initiated. We have noted that tetraethylammonium (.3 - 10 mM) causes a large, dose-dependent contraction of SHR but not WKY aortic muscle (n=8). We conclude that: (1) The same type of voltage- and calcium-dependent K^+ channel exists in WKY and SHR aortic membranes, (2) the opening probability of this 100 pS channel at resting membrane potential is greater in the SHR membrane, and (3) this enhanced opening probability of the SHR channel may be an important intrinsic factor that attenuates vascular muscle excitability in arteries from genetically hypertensive rats.

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Tu-Pos155

MOLE-FRACTION DEPENDENCE OF SLOWING OF TAILS BY Rb^+ IN MIXTURES OF K^+ AND Rb^+ PROVIDE FURTHER EVIDENCE THAT AN EXTERNAL MODULATORY SITE REGULATES CLOSING KINETICS OF TYPE *L* K^+ CHANNELS. by Mark S. Shapiro and Thomas E. DeCoursey, Department of Physiology, Rush Medical Center, Chicago, IL.

External Rb^+ slows deactivation of type *l* K^+ channels by >10-fold in MRL/*lpr* murine lymphocytes studied with the whole-cell patch clamp technique (*Biophys. J.*, 55:200a). The only subtle effects of internal Rb^+ or internal Cs^+, and the lack of effect of raising $[K^+]_o$ from 4.5 to 160 *mM* or of raising $[Rb^+]_o$ from 10 to 160 *mM* on tail kinetics (τ_{tail}) suggest that an external modulatory-site model might explain the effect of Rb^+ on the closing rate better than an "occupancy" type model. We performed whole-cell experiments in which τ_{tail} and the conductance relative to that in all K^+ (τ_{rel} and g_{rel}, respectively) were measured in mole-fractions (*m-f*) of K^+ and Rb^+, keeping $[K^+ + Rb^+]_o$ constant at 160 *mM*. g_{rel} decreased to near g_{Rb} by only small Rb^+ fractions, as though Rb^+ ions permeate type *l* channels more slowly than K^+ ions and so interfere with K^+ permeation. The τ_{tail} *vs. m-f* relationship, however, was strongly concave; i.e. nearly all the K^+ must be replaced by Rb^+ before closing is substantially slowed. In a 1:1 mixture, τ_{rel} averaged only 2.2. In 1 part K^+ to 7 parts Rb^+, τ_{rel} was 5.4, but in all Rb^+ τ_{rel} averaged 14.2, (all at -70 mV). In the 1:1 mixture, on the other hand, g_{rel} was already reduced to 77% of the difference between g_K and g_{Rb}. In an "occupancy" model in which longer binding by Rb^+ *vs.* K^+ during permeation prevents channel closing via a "foot-in-the-door" mechanism, the g_{rel} and τ_{rel} *vs. m-f* relations are expected to roughly correlate, in contrast with our results. The observed concave relation, however, is well-described assuming an external modulatory site, located near the external mouth of the pore (perhaps in an outer 'vestibule'), which binds K^+ and Rb^+ in proportion to their mole-fraction at the site and that when bound by Rb^+ rather than K^+, slows closing 14-fold. *Supported by N.I.H. grants HL01928 and HL37500 (TD).*

Tu-Pos156

PERMEABILITY AND BLOCK IN I_h CHANNELS OF ROD PHOTORECEPTORS.
Lonnie P. Wollmuth & Bertil Hille (Intro. by Charles Stirling), Department of Physiology & Biophysics, University of Washington, Seattle, WA 98195.

We are investigating I_h, an inward current activated by hyperpolarization, in rod photoreceptors of tiger salamanders using whole-cell voltage-clamp. The aim is to define the permeability of I_h channels and to study the effect of various monovalent cations on the channel conductance. The pipette solution contained (mM): 100 KCl; 10 HEPES; 1.5 K_2ATP; 3.5 $MgCl_2$; 1 EGTA; pH 7.4. The reference external solution contained (mM): 20 KCl; 90 TEACl; 8 glucose; 5 histidine; pH 7.4 (HCl). Rods were held at -30 mV, stepped to -110 mV for 200 ms to activate I_h channels and then to various test potentials. I_h was defined as the current blocked by adding 2 mM Cs.

In the 20 mM K reference solution, I_h reversed at -33±3 mV (23 rods, mean±SEM). Lowering the external K concentration to 2 mM (108 mM TEA) shifted the reversal potential for I_h by -61 mV to -94±6 mV (n=3) and the Nernst potential for K by -59 mV. Lowering K also reduced the conductance. After the prepulse to -110 mV, the limiting slope conductance for inward currents was 13±2 nS in 20 mM K (n=4) and 3±1 nS in 2 mM K (n=3). In the absence of external K, the conducance was even less. In 20 mM Na and no K, the slope conductance was 0.3±0.1 nS (n=4). The relative permeability of the channels to monovalent cations was assessed by measuring the change in the reversal potential when 20 mM KCl was exchanged for an equimolar amount of another monovalent cation. Preliminary experiments give the following permeability ratios (P_x/P_K): K, 1; Na, 0.36±0.04, n=5; NH_4, 0.22±0.03, n=4; Li, < 0.07, n=2; methylamine, < 0.07, n=1; tetramethylammonium, < 0.03, n=3. Rb blocked I_h channels, possibly in a manner similar to Cs, but was less potent. Thus although this channel does not distinguish well between Na and K, the block by Rb and Cs and the low permeability to methylamine and Li suggest a small pore size and low field strength.

Supported by NIH grant NS08174, NIH Training Grant GM07108-16 (LPW) and a McKnight Research Award

Tu-Pos157

DEVELOPMENT OF VOLTAGE-GATED POTASSIUM CURRENTS ON RAT SYMPATHETIC NEURONS IN VIVO. Sarah McFarlane and Ellis Cooper. (Intro. by I.W.Hunter) Dept. of Physiology, McGill University. Montréal, Québec. H3G 1Y6.

Little is known about factors that influence the expression of ionic currents on neurons. To learn more, we have been investigating the developmental expression of voltage-gated K currents on superior cervical ganglia neurons, using whole-cell recording techniques. The outward K currents on these neurons are made up of three different components: a non-inactivating current (IK) that activates slowly; a fast transient current (IAf) that activates rapidly and inactivates in 10-30mS; and, a slow transient current (IAs) that inactivates with 2 components, one with a time constant of 150-300ms and the other in 1-3s. The voltage-dependence for activation and inactivation of IAs is 20-25mV more positive than IAf. In neonates, on average 60% of the outward current is made up of IAs (n=45), whereas IAf makes up only 25%, and IK makes up the remaining 15%. In neurons from 2 week old animals, however, the opposite is true: IAf makes up 60% of the outward current, and IAs makes up only 26% of the total current. The contribution of IK remains unchanged.

This change from a predominantly slow transient outward current to a predominantly fast transient outward current is also reflected by changes in current densities of IAf and IAs over the 2wk period. Only 35 of 48 neonatal neurons expressed detectable IAf, and the mean IAf density on these 35 neurons was 30.7pA/pF. By 2wks, the density of IAf increased more than 3 fold to 93.1pA/pF; all 38 neurons from 2wk old animals expressed IAF. In contrast, the density of IAs decreased by more than half: in neonates, the mean density was 97.5pA/pF(n=45), whereas 2wks later the density was only 44.3pA/pF(n=38). The proportion of neurons expressing IAs at both stages remained the same (95%). IK showed little change over this period; the mean IK density was 20.9pA/pF in neonatal neurons and 15.9pA/pF 2wks later. We conclude that a significant change in the expression of IAs and IAf occurs over the first two weeks after birth. The factors responsible for this change are currently being investigated. (Supported by MRC of Canada.)

Tu-Pos158

TEMPERATURE DEPENDENCE OF CHANNEL BLOCK FOR A K^+ CHANNEL IN CULTURED HIPPOCAMPAL NEURONS. Xue-Ping Wang and James McLarnon (Intro. by L.D. Burtnick). Dept. Pharmacology and Therapeutics, The University of British Columbia, Vancouver, B.C. Canada, V6T 1W5.

Non-competitive block of chemically activated ion channels has been reported for a diversity of drugs. In many of these cases, as well as for block of voltage-activated ion channels, the drug actions are subsequent to activation of the channel. Simple bulk occlusion of ion channels by a blocking moiety is often assumed as the basis for channel block. However, there is little evidence available to support this argument. If occlusion were the basis for the channel block, then it would seem reasonable to predict that block by such a process would not be strongly temperature-dependent. In order to test this, we have used a drug (RP-62719) which blocks a calcium-dependent potassium channel in CA1 hippocampal neurons in a manner consistent with the predictions of a simple open sequential channel block scheme. When the bath temperature was lowered by 10°C, the channel transitions from the open state to a non-conducting blocked level were decreased in frequency and the mean open time was significantly increased. The Q_{10} values for the onward (blocking) rate constant of the drug were determined to be near 2.0 and preliminary experiments suggest that the off (channel unblocking) rate constant is also temperature-dependent. These measurements will be relevant to the testing of possible molecular mechanisms which could be involved in the channel block process, including diffusion of the drug to the blocking site and interactions of the drug with the ion channel.

Tu-Pos159

ARE LUMPED MARKOV SYSTEMS STILL MARKOV SYSTEMS?
D.Petracchi Inst. of Biophysics of CNR, Via S.Lorenzo 26, 56127 PISA (Italy).

The general definition of a Markov system of zero order requires that the statistical evolution of the system depends only on its present state. But what does it mean the word "state"? For a point mass moving in a set of potential wells and thermally interacting with the medium, the state is defined when the coordinates and their time derivative are known and then the system evolution depends only on the state. Now we look at this system in a coarse-grained scale disregarding the coordinates and speaking only of open and closed states according to the actually occupied potential well (some wells being classified open and some other closed); can we still consider this system a Markov one? The problem is faced by a computer simulation showing that the system, at this level of knowledge of its state, appears as a complex one with a long lasting correlation with its past history. Another case concerns the Markov schemes of ion channels. The general theory gives the conditional distributions F(t,T) of closings preceded by openings longer than T(θ_i and τ_j being the time constants of open and shut times and N_o and N_c the number of open and closed states:

$$F(t,T)=\sum_{ij}^{N_c} P_j(T)\exp(-t/\tau_j) \quad \text{with} \quad P_j(T)=\sum_{ii}^{N_o} Z_{ij}\exp(-T/\theta_i)$$

The use of these expressions allows a complete reconstruction of the kinetic scheme if only transitions between states with different conductance are allowed. Now what happens if, owing to the signal filtering, brief events are lost? The components of the distributions are still exponentials (although with different time constants) but computer simulations show that the above expression of $P_j(T)$ is no more verified in filtered and lumped sequences of dwell times. Thus the straigthforward reconstruction of the model is no longer possible. Following step by step the simulation it is possible to understand intuitively why this occurs and to select the features of Markov processes still holding in filtered recordings.

Tu-Pos160

USE OF CONDITIONAL DISTRIBUTIONS IN THE ANALYSIS OF ION CHANNEL RECORDINGS. M. Barbi °,M. Pellegrini* ,M. Pellegrino * ,D. Petracchi °, A. Simoni * (Intro. by C. Frediani). ° Inst. of Biophysics CNR, Via S.Lorenzo 26, 56127 Pisa (Italy), * Dept. of Physiology and Biochemistry, Univ. di Pisa, Via S.Zeno 31, 56127 Pisa (Italy).

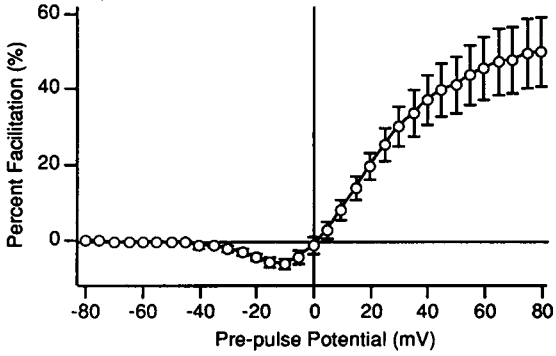
Ion channels have usually been modelled as Markov systems with a few discrete states, thus assuming that the rate constants for leaving a state and reaching any other state are independent of: i) the time spent in the first state and ii) the way this state was reached. This analysis aims to test if a set of experimental data matches these hypotheses. We use in the analysis 1 st order conditional distributions, which are obtained by selecting the dwell times preceded by intervals belonging to given ranges, and 2 nd order conditional distributions, obtained when subsets of openings or closings are selected by limiting the range of the preceding interval of the same kind. Usually, conditioning the previous dwell time does not determine the state occupied by the system, but a given superposition of states. If the state superposition determined by a condition A yields the same 1 st order conditional histogram as a different condition B does, we must expect that also the shapes of the 2 nd order conditional histogram coincide. This is a practical way to test point i) of the hypotheses; to test point ii) it is necessary to show that the exponential fit makes sense and is not a mere computing trick. This last point can be tested if suitable conditions strongly enhance a single component. By applying this strategy to data obtained from a BK channel of mouse EC-1003 cells, we provide evidence that a real physical meaning can be assigned to the longest exponential components of the distributions of open and closed dwell times. Moreover the use of doubly conditional distributions indicates that the system evolution depends only on its actual state.

Tu-Pos161

DOUBLE-PULSE CALCIUM CHANNEL CURRENT FACILITATION IN ADULT RAT SYMPATHETIC NEURONS.

Stephen R. Ikeda. *Department of Pharmacology & Toxicology, Medical College of Georgia, Augusta, GA 30912-2300.*

Double-pulse facilitation of calcium channel currents in enzymatically dispersed adult rat superior cervical neurons was investigated using the whole-cell variant of the patch-clamp technique. Barium currents, elicited by a 0 mV test pulse, were increased in amplitude if preceded by a 40 ms pulse to voltages greater than 0 mV(see figure). The magnitude of facilitation was dependent on pre-pulse voltage and reached a maximum of 50% at a pre-pulse voltage of +80 mV. Control and facilitated activation curves, as derived from tail current amplitudes, were described by the sum of two Boltzmann functions. A facilitating pre-pulse produced an increase in the proportion of the current contributed by the component activated at more hyperpolarized test potentials. The dihydropyridine calcium channel agonist (+)202-791 (1 μM) produced a 24% increase in the barium current amplitude at 0 mV and a slowing of tail current kinetics but produced little change in the magnitude of facilitation. The calcium channel blocker ω-conotoxin GVIA (15 μM) reduced the barium current amplitude to 35% of control at 0 mV. The ω-conotoxin-resistant component was not facilitated by depolarizing pre-pulses. Supported by NIH grant HL-43242 and a grant from the Georgia Affiliate of the A.H.A.



Tu-Pos162

CALCIUM CHANNELS IN C3H/10T1/2 MOUSE FIBROBLASTS: THEIR ROLE IN PDGF-STIMULATED INTRACELLULAR CALCIUM SIGNALS.

Mark Estacion & Lawrence Mordan (Intro. by Martin Rayner), Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

Patch-clamp recordings were obtained from C3H/10T1/2 mouse fibroblasts. When the cells have reached density-dependent growth arrest, a percentage of cells expressed voltage-activated inward currents which persist after exchanging external Na^+ with isotonic Ba^{2+}. The current elicited by 50 msec pulses given from a holding potential of -80 mV shows induction at -40 mV and peaks at -10 mV. At all potentials the current showed complete fast inactivation during the pulse. The voltage-dependence of activation and inactivation suggest that this current shares characteristics of type T calcium channels. PDGF induces in confluent, density-dependent growth arrested C3H/10T1/2 mouse fibroblasts a calcium mobilization with transient and sustained components. The sustained component was dependent upon extracellular calcium and could be selectively blocked by La^{3+}, Diltiazem, Verapamil, or Retinoids. Electrophysiological experiments were performed to see if corresponding modulation of voltage-activated calcium channels could be induced by PDGF. In the majority of experiments, an increase in total inward current was induced by treatment of cells with 10 nM PDGF. The kinetics of the total inward current were not appreciably changed. Experiments are in progress to test the pharmacological properties of the calcium current induced by PDGF stimulation.

Tu-Pos163

Developmental Changes in Calcium Currents of Rabbit Ventricular Cells. Toshiyuki Osaka and Ronald W. Joyner,Department of Pediatrics Emory University Atlanta, GA, (Introduced by Dr. J. Malamud)

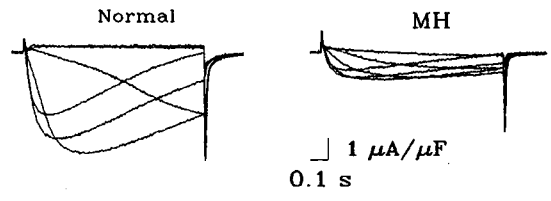
We studied the postnatal development of whole-cell L-type Ca^{2+} current (I_{Ca}) in isolated adult (AD) and newborn (NB) (1-3 days old) cells. I_{Ca} was recorded with Cs^+-rich pipettes and a Na^+-and K^+-free bath solution at 36 degrees C to eliminate other currents. I_{Ca} peak density was higher in AD cells than in NB cells at potential levels between 0 to +50 mV with 1.8 mM Ca^{2+} as the charge carrier. There was no shift in the I-V relationship between AD and NB cells. The maximum I_{Ca} density was 9.9 ± 2.0 pA/pF at 14 ± 5 mV in AD cells (n=11) compared to 5.6 ± 2.0 pA/pF at 13 ± 5 mV in NB cells (n=7) (mean ± SD). Half time ($T_{1/2}$) of inactivation showed a nearly U-shaped relation to membrane potentials from -10 to +30 mV with the shortest $T_{1/2}$ at the potential giving the maximum I_{Ca} density (V_{peak}) in both groups. $T_{1/2}$ at 0 and +10 mV were slightly but significantly longer in NB cells (16.8 ± 4.6 and 13.5 ± 2.4 msec, respectively) than in AD cells (12.6 ± 3.0 and 10.6 ± 1.5 msec). Replacement of Ca^{2+} with Ba^{2+} caused a negative shift in the I-V relationship of about 10 mV in both groups, and an increase in current density with the maximum I_{Ba} density of 18.1 ± 8.1 pA/pF at 1 ± 6 mV for AD (n=8) and 8.6 ± 1.5 pA/pF at -2 ± 4 mV for NB cells (n=5). Ba^{2+} also caused a prominent prolongation of $T_{1/2}$ of inactivation in both groups with a shortening of the $T_{1/2}$ as test potential increased from -10 to +30 mV, suggesting that the inactivation for I_{Ba} is mainly dependent on voltage. The voltage dependency of $T_{1/2}$ of inactivation was not different between the two groups. Along with the result of $T_{1/2}$ for I_{Ba}, there was no difference in the steady-state inactivation of I_{Ca} for AD versus NB cells, suggesting that the shorter $T_{1/2}$ of inactivation for AD cells is mainly due to the increase in a Ca^{2+}-dependent component of I_{Ca} inactivation because of its higher I_{Ca} density. The steady-state voltage dependence of activation of I_{Ca} was also the same for AD versus NB cells. We conclude that L-type I_{Ca} density (pA/pF) increases after the postnatal period without changing the voltage dependent activation and inactivation properties.

Tu-Pos164

REDUCED TRANSVERSE TUBULE Ca2+ CURRENTS IN HUMAN MALIGNANT HYPERTHERMIC SKELETAL MUSCLE.

R. Mejía-Alvarez, M. Fill, J. García, T. E. Nelson*, and E. Stefani. Dept. Molecular Physiology & Biophysics, Baylor College of Medicine, and *Dept. Anesthesiology, University of Texas Health Science Center, Houston, TX.

Individual human skeletal muscle fibers and single human transverse tubule (TT) Ca^{2+} channels from normal and malignant hyperthermic (MH) muscles were studied. Single fibers were dissected free from vastus lateralis biopsied muscle. MH susceptibility was assessed by *in vitro* procedures. Macroscopic dihydropyridine (DHP) sensitive Ca^{2+} current was recorded by using the double Vaseline gap technique at 27°C and 10 mM $CaCl_2$ (external solution). The activity of single DHP-sensitive TT Ca^{2+} channels in the presence of 2.5 μM (±) Bay K was measured in lipid planar bilayers using standard techniques. Fibers and single channels from normal muscle were compared with those from MH subjects. The macroscopic Ca^{2+} current density per capacitance (fig.) was lower in MH fibers than in normal muscle (i. e. at +20 mV: -1.08 ± 0.39 μA/μF and -2.57 ± 0.34 μA/μF respectively; $p < 0.005$). In the presence of 1 μM (±) Bay K the MH current-voltage relationship was shifted by about 15 mV towards more negative potentials (50% activation point = -2.3 ± 2.8 mV and +14.6 ± 3.7 mV for MH and normal respectively). Single DHP-sensitive Ca^{2+} channels from normal and MH patients showed identical conductance (12.1 pS, 100/0 mM $BaCl_2$) and similar open probability (Po = 0.08 ± 0.02 and 0.083 ± 0.03, respectively). Our data indicate that isolated DHP-sensitive Ca^{2+} channels from MH muscle do not have altered single channel properties (Po and conductance). Possible explanations for the reduction of macroscopic Ca^{2+} current observed in MS single fibers are, 1) TT from MH muscle contain fewer functional Ca^{2+} channels, 2) the unusually high resting $[Ca^{2+}]_i$ may promote a Ca^{2+}-dependent inactivation, and/or 3) down regulation of MH Ca^{2+} channels as a pathological response to decrease Ca^{2+} entry from the extracellular fluid. Supported by MDA (J.G.), NIH AR01834 (M.F.) and AR38970 (E.S.).



Tu-Pos165

PURIFICATION AND FUNCTIONAL RECONSTITUTION OF A BOVINE BRAIN RYANODINE RECEPTOR. F. Anthony Lai, Le Xu, Gouri Kumari, Hee-Bong Lee and Gerhard Meissner. Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260.

Previous ryanodine binding and single channel measurements have indicated the existence of a ryanodine receptor channel in brain (Ashley, *J. Membr. Biol.* 111, 179; Lai *et al.*, *Biophys. J.* 57, 529a). The inositol trisphosphate receptor, another intracellular calcium release pathway, has been shown to be enriched in the cerebellum (Supattapone *et al.*, *JBC* 263, 1530). In contrast, our ryanodine binding studies with membranes prepared from bovine cerebellum and bovine whole brain minus cerebellum (WB-C) have revealed a significantly lower concentration of ryanodine binding sites in cerebellum (< 0.02 versus 0.25 pmol/mg protein in WB-C). [^{3}H]Ryanodine binding studies indicated the presence of a high affinity (K_D of ~3 nM) and lower affinity sites in WB-C microsomes. The Chaps-solubilized ryanodine receptor from bovine WB-C was purified as a 30S protein complex on sucrose gradients, and shown by SDS PAGE to comprise a single high molecular weight polypeptide, which migrated with a mobility greater than that of the rabbit skeletal ryanodine receptor and corresponding to that of the canine cardiac receptor (Lai *et al.*, *BBRC* 151, 441). Immunoblot analysis of WB-C microsomes and 30S gradient fractions showed a cross-reaction with monoclonal antibodies raised against the purified canine cardiac muscle ryanodine receptor. These studies suggest that the brain ryanodine receptor is, in several respects, similar to the cardiac muscle ryanodine receptor, and is present in very low abundance in cerebellum in contrast to the inositol trisphosphate receptor. Supported by USPHS and MDA grants.

Tu-Pos166

HIGH-SPEED IMAGING OF BAY K 8644-INDUCED CALCIUM INFLUX SUGGESTS THAT CERTAIN CALCIUM CHANNELS MAY BE POLARIZED IN THE CELL MEMBRANE OF THE RAT GONADOTROPE.

S.R. Rawlings, D.J. Berry & D.A. Leong. (Intro. by J.T. Hackett) Dept. Medicine, University of Virginia, Charlottesville, VA 22908.

Luteinizing hormone-releasing hormone (LHRH) stimulates a biphasic increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in anterior pituitary gonadotropes. The first "spike" phase reflects the Ins(1,4,5)P_3-mediated mobilization of Ca^{2+} from intracellular stores, while the second "plateau" phase is believed to result from influx of Ca^{2+} into the cell through dihydropyridine (DHP)-sensitive Ca^{2+} channels. Previous work using the fluorescent Ca^{2+}-indicator fura-2 and high-speed (one image every 33 ms) video-imaging microscopy revealed that the spike phase of Ca^{2+} mobilization consisted of a wave of Ca^{2+} that began at a specific "initiation" site in the cell. The DHP-sensitive Ca^{2+} channel agonist BAY K 8644 (BAYK) was used in the present study to image the influx of Ca^{2+} into these cells.

BAYK (10^{-8}-10^{-5}M) stimulated either a sustained rise in $[Ca^{2+}]_i$, or a series of Ca^{2+} oscillations in acutely dispersed rat gonadotropes. These responses were blocked in the absence of extracellular Ca^{2+}. High-speed imaging revealed waves of Ca^{2+} that underlied the BAYK-induced Ca^{2+} oscillations. These Ca^{2+} waves originated either from a defined point in the cell membrane (a "polarized" wave), or as an evenly distributed rise in Ca^{2+} around the cell border (a "radial" wave). Of six cells analyzed, three showed a clear polarization of Ca^{2+} influx, one exhibited only radial waves, and two showed both polarized and radial waves. In all cases the polarized Ca^{2+} influx was spatially distinct from the site of LHRH-induced Ca^{2+} mobilization from intracellular stores.

These findings are the first to demonstrate that Ca^{2+} influx, and thus DHP-sensitive Ca^{2+} channels, may be polarized in endocrine cells. Since the influx of Ca^{2+} is an important step leading to hormone secretion, localized Ca^{2+} influx may be a critical factor in determining the polarization of hormone secretion recently demonstrated in other endocrine cell types.

Tu-Pos167

CHARACTERIZATION OF THREE BACKGROUND CALCIUM CHANNELS IN ADRENAL GLOMERULOSA CELLS. T. DURROUX[1], N. GALLO-PAYET[2], M.D. PAYET[3]. [1]Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, 34094 Montpellier Cedex 5, FRANCE. ; [2]Département de Médecine, Service d'Endocrinologie ; [3]Département de Physiologie-Biophysique, Faculté de Médecine, Université de Sherbrooke (Qué) J1H-5N4, CANADA.

A background Ca^{2+} influx sensitive to variations in extracellular calcium concentration and a basal secretion of aldosterone and corticosterone have previously been detected in adrenal glomerulosa cells. Recently, we have described three calcium channels (Durroux et al (1988) J. Physiol. 404, 713-729) -named T, L and N- in rat adrenal glomerulosa cells but none of these channels can be responsible for the background calcium influx. The cell-attached recording mode of the patch-clamp technique was used to characterize background calcium currents. With a pipette filled with 110 $BaCl_2$ or 90 $CaCl_2$, we described three background calcium channels, named B_1, B_2 and B_3. The B_1-type demonstrates a non-linear I-V curve with a zero current voltage close to +50 mV and conductances of 4 and 7 pS at -40 and -70 mV, respectively. The opening probability p_o vs. membrane potential is bell-shaped with its maximum at -70 mV. The conductance of the B_2-type is 6.6 pS while the B_3-type shows a non-linear I-V relationship with conductances close to 16.1 and 10 pS at HPs of -60 and -20 mV. These three types of current are not sensitive to nifedipine (5.10^{-5}M).

We suggest that these background currents could be responsible for the basal calcium influx and aldosterone secretion previously observed in non-stimulated glomerulosa cells.

Tu-Pos168

VOLTAGE-SENSITIVE CALCIUM CURRENTS IN CULTURED PURKINJE NEURONS. C.R. Deal and D.L. Gruol, Res. Inst. Scripps Clinic, La Jolla, CA 92037.

Ca^{2+} currents in Purkinje neurons (PNs) were studied under whole cell recording conditions using the single electrode voltage clamp in the switching mode. PNs with limited dendritic structure from 2 to 3 week old cultures were used. A contribution of Na+, K+ and Cl- currents was minimized by pharmacological blockade and ion substitution. All neurons studied (n=27) expressed a high threshold (HT) Ca^{2+} current with transient and sustained components and a rapidly inactivating low threshold (LT) Ca^{2+} current. When the holding potential (hp) was -90 mV, the LT current was first evident at test potentials (tps) around -40 mV. The HT component was evident at tps around -20 mV. Maximal currents were observed at tps around ~0 mV and appeared to include both HT and LT components. When the hp was -63 mV, the normal resting potential of the PNs, the HT and LT currents were evident at tps similar to that observed at the -90 mV hp. At a hp of -40 mV, only the sustained component of the HT current was evident. The HT component was potentiated by barium (4mM) and antagonized by cadmium (50 μM). The LT component was relatively resistant to these agents. Nickel (200 μM) and amiloride (100 μM) had variable effects on the LT and HT currents. These data indicate that PNs possess at least 2 Ca^{2+} conductances. The pharmacological profile of these conductances varies somewhat from that observed in peripheral neurons. Supported by NS21777.

Tu-Pos169

FLUCTUATION ANALYSIS OF CALCIUM CHANNELS OF MURINE HIPPOCAMPAL NEURONS. J.D. Prasad, G.-J. Huang, S. Laxminarayan, and J.J. McArdle. N.J. Inst. Technology and Depts. Pharmacol. & Acad. Comp. Center, UMDNJ-N.J.Med.Sch. Newark, N.J. 07103

In an attempt to estimate the single channel conductance and open time of calcium channels of hippocampal neurons isolated from adult mice and bathed in a buffer containing physiologic levels of divalent cations, we subjected the whole cell calcium current (I_{Ca}) to power spectral density (PSD) analysis. To do this, neurons were voltage-clamped to a V_h of -70 mV and then stepped (150 mSec) to +30 mV in 10 mV increments. We then collected 25 currents in response to a V_c (2 Sec) which produced an I_{Ca} whose peak amplitude during the linear portion of its decay was approximately 10% of the cell's maximum I_{Ca}. The time between the 25 current traces was approximately 20 Sec. This and the presence of ATP (4 mM) in the recording pipette prevented significant rundown of I_{Ca}. Each current trace was preceded by a V_c which did not activate I_{Ca} but provided background noise. Currents were sampled at 1 Khz and low pass filtered at 300 Hz. A block of 1024 points was taken from the linear tail portion of each current and passed through appropriate filters to eliminate the presence of any possible linear trends. Next, the experimental and background data were divided into two sub-blocks prior to Fast Fourier Transformation. Double logarithmic plots of PSD were made after applying frequency domain smoothing. The final difference spectrum, obtained after subtracting each background from the corresponding experimental and then averaging, was fitted by a Lorentzian function to enable estimation of channel kinetics and conductance. The implications of the results will be discussed. (NIAAA grant R01 AA08025)

Tu-Pos170

PERMEATION OF DIVALENT CATIONS THROUGH THE Ca^{2+} CHANNEL OF PORTAL VEIN (PV) MYOCYTES

D. Katzka, R. Cox, A. Davidoff and M. Morad, Dept.'s of Medicine and Physiology, Univ. of Pennsylvania, Philadelphia, PA 19104

Ca^{2+} channels are characterized by their high selectivity to Ca^{2+} and divalents over monovalents. We examined the divalent selectivity of the Ca^{2+} channel in rabbit PV by the whole cell clamp method. When Ca^{2+} was replaced by Ba^{2+} or Sr^{2+}, the order of maximum current was $Ca^{2+}=Ba^{2+}>Sr^{2+}$ at 2 mM, $Ba^{2+} > Sr^{2+} \geq Ca^{2+}$ at 5-10 mM. We examined whether this concentration-dependent difference in divalent ion selectivity resulted from contaminant Ca^{2+} block of Ba^{2+} and Sr^{2+} currents. Myocytes were dialyzed with a solution containing (in mM): CsCl 120, NaCl 10, TEACl 20, Mg-ATP 5, EGTA 14, HEPES 10 at pH 7.2. Addition of 500 μM EGTA increased maximum I_{Ba} or I_{Sr} by 10-100% suggesting that contaminant Ca^{2+} significantly blocked these currents. Since previous studies of the anomalous mole fraction used mM Ca^{2+}, we examined whether a decrease in I_{Ba} with mM Ca^{2+} resulted from a masked blocking effect of μM Ca^{2+}. Currents through the Ca^{2+} channel were recorded in solutions with varying Ca^{2+} and Ba^{2+} keeping total divalents constant at 5 mM. In contrast to prior studies, μM amounts of Ca^{2+} when added to Ba^{2+} solutions (5 mM Ba, 10 μM EGTA) attenuated the current. I_{Ba} through the Ca^{2+} channel was decreased by 6% by 10 μM Ca^{2+}, 14% by 20 μM, 22% by 50 μM, and 33% by 100 μM Ca^{2+} ($n \geq 4$). Within this $[Ca^{2+}]_o$ range there was no significant change in the kinetics of I_{Ba} suggesting little or no permeation of Ca^{2+}. I_{Ba} decreased to 41% of its maximum when Ba:Ca ratio was 4:1 and increased to 62% at Ba:Ca ratio of 0:5. Within this $[Ca^{2+}]_o$ range, however, kinetics of inactivation changed significantly with varying $[Ca^{2+}]_o$. Thus, the PV myocyte Ca^{2+} channel is highly selective to Ca^{2+} such that μM Ca^{2+} can block the permeation of other divalents through the channel, and modify the divalent cation selectivity ratio when based strictly on ion substitution. (Supported by NIH grants HL 16152 and DK 01839.)

Tu-Pos171

CHARACTERISTICS AND SPECIFICITY OF Ca^{2+} CHANNEL BLOCK BY METHYLMERCURY IN PHEOCHROMOCYTOMA CELLS. T. J. Shafer and W. D. Atchison. Dept. of Pharm./Tox., Michigan State Univ., E. Lansing, MI 48824.

Interactions of methylmercury (MeHg) with voltage-sensitive Ca^{2+} channels in rat pheochromocytoma (PC12) cells were examined by measuring effects of MeHg on whole cell Ba^{2+} currents. Specificity for block by MeHg of Ca^{2+} channels was tested by comparison with its ability to block I_{Na} in N1E-115 neuroblastoma cells. Rate of block of I_{Ba} by MeHg increased in a concentration-dependent manner between 1 and 20 μM. Increasing the frequency of stimulation from 0.1 to 0.4 Hz facilitated block of I_{Ba} by MeHg, but channel use was not required for the blocking action of MeHg, as a 2 min application of 10 μM MeHg in the absence of stimulation reduced I_{Ba} by approximately 80%. When the membrane potential was held at -40 mV, block of I_{Ba} by MeHg was voltage-dependent, whereas at holding potentials of -70 and -90 mV, block of I_{Ba} by MeHg was not strongly voltage-dependent. The magnitude of block of Ca^{2+} channels by MeHg depended on the extracellular concentration of Ba^{2+}; decreasing the extracellular concentration of Ba^{2+} from 20 mM to 10 mM increased the magnitude of block by MeHg from 45.6 to 77.3%. Block of I_{Ba} by MeHg was not reversed by washing with MeHg-free solution. The ionic selectivity of PC12 cell Ca^{2+} channels was $Ca^{2+} = Sr^{2+} > Ba^{2+}$. In the presence of MeHg, all three divalent cations were equally permeant. In N1E-115 neuroblastoma cells, 10 μM MeHg caused only small reductions in I_{Na}. These results suggest that: 1) MeHg blocks N- and L-type Ca^{2+} channels in PC12 cells at low micromolar concentrations in a manner which is voltage- but not state-dependent; 2) MeHg alters the ionic selectivity of Ca^{2+} channels; 3) Block by MeHg is not reversed by washing with MeHg-free solutions, but is antagonized by divalent cations; 4) The effects of MeHg are preferential for Ca^{2+} channels compared to Na^+ channels.

(Supported by NIH grant ES03299. TJS is the recipient of a student fellowship from Hoffman-LaRoche, Inc.)

Tu-Pos172

NIFEDIPINE INHIBITED T CHANNEL CURRENTS: STUDIES ON THE SOLVENT EFFECT. Lingyun Wu, Edward Karpinski, Rui Wang and Peter K.T. Pang. (Intro. by Joy Steele). Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

Previous studies have shown that different solvents modified the actions of dihydropyridines on both T and L channel currents. In the present studies, the solvent effect on the action of nifedipine on T channel currents was further explored. Typical T channel currents were recorded in N1E-115 neuroblastoma cells using the whole cell patch clamp technique. Nifedipine (100 μM) dissolved in acetone or ethanol (EtOH) had no effect on T channel currents. However, nifedipine (10 μM) dissolved in dimethylsulfoxide (DMSO) or polyethylene glycol inhibited T channel currents by 50% ($n=7$, $p<0.05$) and 32% ($n=3$, $p<0.05$), respectively. In the second set of experiments, nifedipine was first dissolved in DMSO at a concentration of 30 mM and then dried by using a flash evaporator at 50°C for 24 hr in the dark. The dried nifedipine was then re-dissolved in acetone or EtOH. It was found that nifedipine re-dissolved in acetone decreased the T channel current by 86% (100 μM, $n=6$, $p<0.05$) and 33% (10 μM, $n=4$, $p<0.05$). Nifedipine re-dissolved in EtOH (100 μM) also inhibited the T channel current by 82% ($n=5$, $p<0.05$). The effect of nifedipine dissolved in DMSO on T channel currents was established 5 min after the addition of the agent. However, at least 15 min was needed for the maximum effect of nifedipine re-dissolved in either acetone or EtOH. The inhibitory effect of nifedipine re-dissolved in either acetone or EtOH on T channel currents could be reversed by wash-out procedures. These results indicate that the molecular conformation of nifedipine might be modified by DMSO so that when subsequently dissolved in acetone or EtOH nifedipine still could inhibit T channel currents. The delay of the action of nifedipine re-dissolved in acetone or EtOH suggests that these two solvents may also exert some effects on nifedipine.

Tu-Pos173

TWO TYPES OF VOLTAGE-DEPENDENT INACTIVATION FOR THE "T TYPE" Ca CURRENT OF INSULIN SECRETING CELLS ?

T.G. Hales and B. Ribalet, Depts. of Anesthesiology and Physiology, University of California Los Angeles, Los Angeles, CA. 90024.

Two types of Ca current were found in insulin secreting cells (HIT), under whole-cell voltage clamp. With 3 mM $CaCl_2$ in the bath, 115 mM N-methyl-D-glucamine chloride and 5 mM BAPTA in the pipette and a holding potential of -100 mV, whole-cell currents, evoked by 100 msec steps to potentials between -50 and 20 mV, comprised transient and sustained current phases. Conditioning pulses of more than 1 s duration to -30 mV inhibited the transient currents. The residual sustained currents activated at -30 mV and were maximal at 0 mV; being sensitive to 0.2 uM nifedipine and larger when Ca^{2+} was substituted by Ba^{2+}, they were identified as "L type" currents. The transient currents, analyzed after subtracting traces recorded with and without conditioning pulses, had "T type" current properties; they were smaller when Ca^{2+} was substituted by Ba^{2+}, were unaffected by 0.2 uM nifedipine, but almost completely blocked by 5 uM. The rates of activation and inactivation of the transient currents increased between -50 mV and -10 mV. The inactivation occurred with BAPTA present, was not correlated with the peak current, and was independent of the type or concentration of divalent cation, suggesting that it is voltage- and not Ca^{2+}-dependent. While the rate of inactivation increased up to -10 mV, it decreased for more positive potentials resulting in sustained currents above 20 mV. To confirm the role of non-inactivating "T type" currents in these sustained currents, a protocol with prepulses and test pulses of 50 msec was used. When transient "T type" currents were elicited by prepulses to -10 mV, test pulses to -10 mV did not evoke transient currents, implying that the prepulse inactivated the current. However, with prepulses to 40 mV, which elicited small but sustained currents, test pulses evoked transient currents, indicating that the prepulse did not inactivate the current. It may be concluded that the "T type" current exhibits fast inactivation which follows a bell-shape curve with minima at -45 and 40 mV and a maximum at -10 mV; this is distinct from the slowly developing inactivation which initially occurred with conditioning pulses to -60 mV and was maximum above -30 mV. (Supported by NSF and ADA).

Tu-Pos174

PERMEATION AND GATING PROPERTIES OF THE L-TYPE Ca CHANNEL IN MOUSE PANCREATIC β-CELLS

Clare Fewtrell, Paul A. Smith and Frances M. Ashcroft
University Laboratory of Physiology, Oxford, England.

Single Ca-channel currents are commonly recorded with high concentrations of Ba in the extracellular (pipette) solution to increase the amplitude of the single-channel current and facilitate its measurement. In many cases a dihydropyridine Ca-channel agonist is also used to prolong channel openings. It is difficult to relate the voltage-dependence of Ca-channel currents recorded under these conditions to those under normal physiological conditions because both the high divalent cation concentration and the channel agonist produce shifts in the voltage-dependence of permeation and gating. To quantify these shifts, we have measured single Ca-channel currents at different Ba concentrations and whole-cell currents at physiological Ca concentrations or in the presence of Ba and Bay K8644.

Ba currents through single L-type Ca channels were recorded from cell-attached patches on isolated mouse panceatic β-cells. The pipette solution contained (mM): 100 $BaCl_2$, 10 TEACl, 10 HEPES (pH 7.4 with NaOH); different Ba concentrations were obtained by substitution with NaCl. The extracellular solution contained (mM): 115 KCl, 1.1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES (pH 7.4 with KOH), 20 glucose and 1μM Bay K8644.

The relationship between single channel conductance (γ) and $[Ba]_o$ saturated with increasing $[Ba]_o$ and could be fitted by the relationship $\gamma = \gamma_{max}/(1+(k/[Ba]_o))$, where the maximal conductance (γ_{max}) is 22pS and the Ba concentration at which γ is half-maximal (k) is 5mM. In 100mM $[Ba]_o$, a shift of +42 ± 2mV (mean ± s.e.m.; n=7) was required to fit the GHK equation to the current-voltage (I-V) relationship, due to binding and screening of membrane surface charge. This shift varied with $[Ba]_o$ and decreased to +26 ± 2mV in 10mM Ba (n=10). The voltage-dependence of channel activation also varied as a function of $[Ba]_o$. Single channel currents could be recorded at membrane potentials as negative as -90mV with 10mM $[Ba]_o$, whereas in 100mM $[Ba]_o$ depolarizations > -50mV were needed to elicit channel openings.

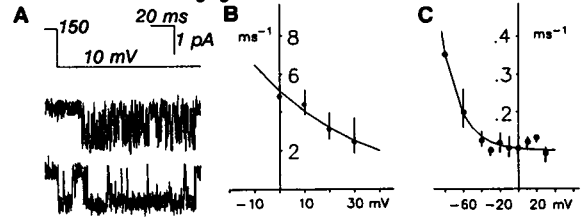
Perforated-patch recordings of whole-cell Ca currents revealed that substitution of a physiologcal solution (2.6mM Ca) by a 10 mM Ba solution containing Bay K8644 produced little or no shift in the I-V relationship. This suggests that the voltage-dependence of single Ca-channel currents determined with 10mM $[Ba]_o$ should be comparable to that found under physiological conditions.

Tu-Pos175

VOLTAGE DEPENDENCE OF CLOSURE FROM SHORT AND LONG-LASTING OPENINGS OF L-TYPE Ca CHANNELS

John P. Imredy and David T. Yue
Dept. of Biomedical Engineering, Johns Hopkins University, Baltimore, MD

One of the emerging paradigms in the gating of various voltage-gated channels is that most of the voltage dependence resides in activation, and that channel transitions surrounding the open state are relatively voltage independent (e.g., A-type K channels [Zagotta & Aldrich, 1990], Na channels [Bezanilla and Armstrong, 1977], T-type Ca channels [Chen & Hess, 1990]). This recurring motif of voltage dependence may arise as a consequence of common structural features of putative voltage sensing regions (S4) among these channels. We are investigating whether L-type Ca channels share a similar pattern of voltage dependence. The question here is complicated by the presence of at least two open states: a short-lived open state ("mode 1" type), and a long-lasting open state ("mode 2" type). Moreover, the frequency of long-lasting openings is often quite sporadic. We took advantage of the findings that 1 mM 8-Br-cAMP and positive voltage prepulses elicit numerous clusters of both types of openings in cell-attached patches from heart cells (A). With 160 mM Ba as charge carrier, the signal-to-noise ratio was sufficient to determine whether closing rates from the two sorts of opening were voltage dependent. Closing rates from short openings, as gauged from fast open time constants, show a simple exponential voltage dependence (B), consistent with a single exit pathway with an equivalent charge movement of .6 e^-. Closing rates from mode 2 openings, as calculated from slow open time constants, manifest greater voltage dependence at more negative voltages (C, < -40 mV), but display a broad voltage-independent plateau at more positive potentials. These features suggest a scheme in which there are two exit routes from the long-lasting open state: one voltage-dependent transition to a closed state traversed during activation (equivalent charge of 1.2 e^-), and a voltage-insensitive path to another closed state present in mode 2. The solid curve in C plots the fit of such a model. Despite the clear-cut voltage dependence of closure from both short and long-lasting openings, the associated equivalent charge movements of these are far smaller than the 5-6 e^- moved during activation (Bean & Rios, 1989). Thus, L-type Ca channels appear to preserve the common pattern of voltage dependence found so far in other voltage-gated channels.

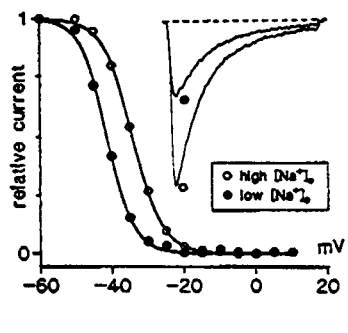


Tu-Pos176

EXTRACELLULAR Na^+ ALTERS THE GATING OF CARDIAC Ca^{2+} CHANNELS IN MAMMALIAN MYOCYTES.

A. Orkand, G. Callewaert, A. Chawla and M. Morad. Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104 and M.D.I.B.L., Salsbury Cove, ME 04672.

Cardiac Ca^{2+} channels are often studied when I_{Na} is blocked by reducing $[Na^+]_o$. The possibility of a direct effect of $[Na^+]_o$ on I_{Ca} was explored in whole cell voltage-clamped, rat and guinea pig ventricular cells. The cells were dialyzed with a solution containing (in mM): 45 NaCl, 80 CsCl, 10 EGTA, 5 Na_4BAPTA, 0.1 cAMP, 0 to 0.1 GTP-γ-S, 2 MgATP, 25 Hepes pH 7.4. The standard external solution contained (in mM): 140 NaCl, 2 $CaCl_2$, 2 CsCl, 1 $MgCl_2$, 10 HEPES, 0.002 TTX at pH 7.4. In low Na^+ solutions, Na^+ was replaced by Cs^+. I_{Ca} activated from -40 mV to 0 mV was reversibly reduced without significant change in the time constant of its inactivation when cells were exposed to 5mM Na^+ containing solutions (inset). Reduction of Na^+ shifted the steady-state inactivation (SSI) of I_{Ca} by 10 mV toward more negative potentials, but had no effect on the voltage-dependence of its activation. The negative shift in SSI was also observed when Ba^{2+} was the charge carrier or when Na^+ was replaced by Li^+. We conclude that suppression of I_{Ca} by low $[Na^+]_o$ is caused by a shift in the voltage dependence of the inactivation process and that this is due, not to a general surface charge effect, but to a specific interaction between Na^+ and the Ca^{2+} channel. (NIH HL 16152)



Tu-Pos177

MODULATION OF CARDIAC L-TYPE CALCIUM CHANNEL GATING BY CALCIUM AND HYDROGEN IONS: EVIDENCE FOR INDEPENDENT BINDING SITES.

Yiu Wa Kwan and Robert S. Kass, Department of Physiology, University of Rochester, Rochester NY 14642.

Previous studies have shown that extracellular calcium (Ca_o^{++}) and hydrogen (H_o^+) ions can independently shift voltage-dependent gating of heart L-type channels in a manner consistent with screening of and binding to negative surface charges. The present study was designed to test for the independence of these binding sites by studying gating shifts when both Ca_o^{++} and H_o^+ were modified. Inactivation of L-type calcium current was measured at room temperature (20-24°C) in guinea pig ventricular myocytes using patch clamp methods in the whole cell configuration. At pH 7.4, calcium-dependent depolarizing shifts in inactivation were measured relative to 5 mM Ca_o^{++} and found to increase with Ca_o^{++} over a concentration range of 5 to 40 mM. No further shift was measured when Ca_o^{++} was elevated to 60 mM (40 mM, 15.9±1.3 mV; 60 mM, 17.1±0.8 mV; (n=3, $p<0.05$). These results suggested that the negatively charged calcium binding sites were saturated at 40 mM Ca_o^{++} We then measured H_o^+-induced gating shifts when Ca_o^{++} was varied systematically and found that the H_o^+-induced gating shifts were affected by Ca_o^{++}. For a fixed change in extracellular (pH_o) (7.4 to 6.0), pH_o-induced shifts ranged from 10 mV to 13 mV when Ca_o^{++} was between 5 and 20 mM (5 mM 13±4 mV, n=5; 10 mM, 10.4±1.9mV, n=5; 20 mM, 11.42± 2.1 mV n=5). When Ca_o^{++} was either 40 or 60 mM, conditions in which Ca_o^{++}-induced gating shifts had saturated, changing pH_o from 7.4 to 6.0 caused additional gating shifts. However in these Ca_o^{++} concentrations, the gating shifts were only 6.3 ± 1.9 mV (Ca_o^{++} -40 mM) and 4.9 ± 2.7 mV (Ca_o^{++}=60 mV). All of these results are consistent with predictions of surface potential theory in which Ca_o^{++} and H_o^+ bind to independent sites.

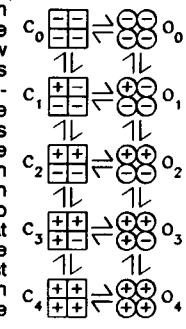
Tu-Pos178

AN ALLOSTERIC MODEL FOR CALCIUM CHANNEL GATING AND DIHYDROPYRIDINE AGONIST ACTION. Theodore N. Marks and Stephen W. Jones. Department of Physiology and Biophysics, Case Western Reserve Univ., Cleveland, OH 44106.

We propose that gating of a voltage-dependent channel is analogous to activation of an allosteric enzyme, and that the MWC model (Monod et al., *J. Mol. Biol.* 12:88-118, 1965) is a useful starting point for description of channel kinetics. On this approach, movement of a voltage sensor is analogous to binding of a ligand, and channel opening corresponds to the global conformational change upon enzyme activation.

In the case of strong cooperativity, the MWC model reduces to C_0-C_1-C_2-C_3-C_4-O_4 (assuming 4 voltage sensors), with the final C-O transition insensitive to voltage, as found recently for potassium channels (Koren et al., *Neuron* 4:39-51, 1990; Zagotta and Aldrich, *J. Gen. Physiol.* 95:29-60, 1990). We find that the C-O transition is not detectably voltage dependent for L-type calcium channels in the A7r5 smooth muscle-derived cell line, using a modified beta distribution analysis of single channel gating. The rate constants for channel opening and closing within a burst are extremely fast (> 4000 s^{-1} and > 2000 s^{-1} respectively). Single channel and whole-cell data are fitted adequately by the C_0-C_1-C_2-C_3-C_4-O_4 model with the constraint that the voltage sensors are identical and independent.

With dihydropyridine (DHP) calcium channel agonists, open time distributions are fit by two exponentials, and fast and slow openings are intermingled. The open times are not detectably voltage- or concentration-dependent. DHP agonists also shift the activation curve to more negative voltages with an increase in slope, and reduce the latencies to first opening. These results can be explained by the full MWC model, with DHP agonists acting as allosteric effectors to shift the C-O equilibria to the right, so that some channels open with only 3 of 4 voltage sensors moved. On this model, the apparent voltage-dependence of DHP agonist action results from the allosteric coupling of voltage sensor movement to channel opening.



Tu-Pos179

'TWO-SITE' BEHAVIOR FROM A ONE SITE MODEL OF THE CALCIUM CHANNEL. C.M. Armstrong, Dept. of Physiology, University of Pennsylvania, Philadelphia, Pa. and Jacques Neyton, Ecole Normale Superieure, Paris, France.

Open calcium channels have several properties that have led to a two-site model for the channel, one site at either end (Hess & Tsien, Almers & McCleskey). Among the properties explained by the model are saturation of current magnitude as a function of calcium (or barium) concentration, monovalent cation conduction in the absence of divalent cations, suppression of monovalent conductance by even a very low concentration of calcium, and, in solutions containing approximately 10 mM barium, interference with barium current by low concentrations of calcium (the 'anomalous mole fraction' effect, AMFE). Stimulated by Robert Chow's analysis of cadmium block of calcium channels, we examined the applicability of a one-site model to these phenomena. A basic tenet of the two-site model is that an ion can enter a site only if it is vacant. This assumption, although reasonable for a neutral site, is inappropriate for a charged site. Instead, a charged site is (almost) never vacant, and conduction involves ion-exchange, or 'knock-on'. All of the phenomena cited can be explained by a one-site channel, in which the site has a charge of -2e. A second divalent cation can approach an occupied site (repulsion by the occupying cation is balanced by attraction to the site), and saturation is related to affinity for the second cation. For such a site, a monovalent cation can seldom replace a divalent, resulting in no monovalent conduction unless divalent concentration is very low. Finally, a small, relatively tightly-bound cation (calcium) is not very likely to be replaced by a larger ion (barium), resulting in the AMFE.

Tu-Pos180

TRYPSIN INCREASES L-TYPE CALCIUM CURRENT IN A7R5 SMOOTH MUSCLE CELLS, AND REMOVES VOLTAGE- BUT NOT Ca^{2+}-DEPENDENT INACTIVATION. C. Obejero-Paz, S. W. Jones & A. Scarpa (Intro. by J. Whittembury). Dept. Physiol. & Biophys., Case Western Reserve Univ., Cleveland, OH, 44106.

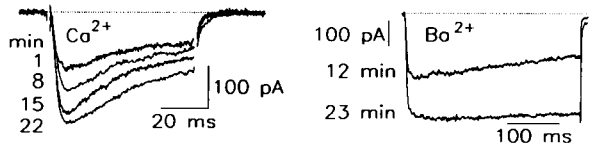
We used the whole cell patch clamp technique to characterize the effect of intracellular trypsin on the L-type calcium channel in the A7r5 cell line. Trypsin (50-100 μg/ml) increased currents carried by either Ca^{2+} and Ba^{2+} by a factor of 1.35-3. This effect was inhibited by trypsin inhibitors.

In control conditions, inactivation of currents in Ba^{2+} can be fitted by the sum of one constant component and an exponential function with a time constant of several hundred msec. In 6 cells, trypsin almost completely removed inactivation of currents carried by Ba^{2+}. This effect can explain part, but probably not all, of the increase in current caused by trypsin.

In control conditions, inactivation in Ca^{2+} can be described by the sum of one constant factor and two exponential functions. With trypsin, the more rapid component of inactivation in Ca^{2+} was similar to control at all potentials studied: at +20 mV, control τ = 26 ± 4 msec, and trypsin modified current τ = 24 ± 5 msec (mean ± STD). The rapid component of inactivation was current-dependent both in control and after trypsin treatment. The slower component of inactivation in Ca^{2+}, which appeared to be voltage-dependent, was reduced by trypsin.

These results are consistent with the existence of distinct protein domains for Ca^{2+}-dependent and voltage-dependent inactivation of calcium current. The trypsin sensitivity of voltage-dependent inactivation of sodium channels, A-type potassium channels, and L-type calcium channels might suggest a common molecular mechanism.

Supported by NIH grant HL41206. C. O.-P. is a Research Fellow of the American Heart Assoc., Northeast Ohio Affiliate, Inc.



Tu-Pos181

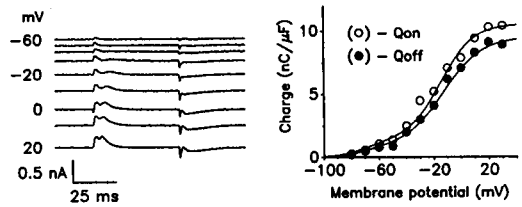
KINETIC COMPONENTS OF INTRAMEMBRANE CHARGE MOVEMENT IN GUINEA-PIG VENTRICULAR MYOCYTES. R. Shirokov, R. Levis, I. Stavrovsky, and E. Ríos. Rush University, Chicago, IL.

Non-linear capacitative current was studied in enzymatically isolated guinea-pig myocytes under conditions similar to those of Bean & Ríos (1989) and Hadley & Lederer (1989). With low-resistance patch pipettes and series resistance compensation, cell capacitance was charged in about 0.5 ms. In 8 of 36 cells, a slow component with a prominent rising phase was visible in the asymmetric ON current (Figure). In most cases the OFF current had two kinetic components, but in 4 cells there were three OFF components. The fast OFF component had a time constant of about 1 ms, started to activate at potentials from -80 mV and was reduced at holding potential (H.P.) -40 mV. The slow one had a time constant of 8 ms and activated at -50 mV and higher potentials. At H.P. -110 mV the voltage dependence of total charge was well fitted by the sum of two Boltzmanns. The average values of parameters were Q_{max1} = 1.6 nC/μF, V_{t1} = -70 mV, K_1 = 10 mV and Q_{max2} = 11.6 nC/μF, V_{t2} = -10 mV, K_2 = 13 mV.

Presumably the two Boltzmann components in the voltage dependence correspond to the two kinetic components and respectively to Na and Ca gating currents.

The possibility that some kinetic feature of the current is due to inhomogeneous clamping will be explored by independent measurements of intracellular potential in two-pipette experiments.

Supported by NIH and AHA.



Tu-Pos182

EFFECT OF PHOTORELEASE OF INTRACELLULAR CA ON CA CURRENT AND CA CHANNEL GATING CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES. Robert W. Hadley & W.J. Lederer, Dept. of Physiology, School of Medicine, Univ. of Maryland, 655 W. Baltimore St., Baltimore, MD 21201.

L-type Ca currents and Ca channel gating currents were studied using the whole-cell patch-clamp technique under appropriate ionic conditions in guinea-pig ventricular myocytes. $[Ca]_i$ could be transiently elevated during an experiment through UV illumination of the photolabile Ca chelator DM-nitrophen, which was introduced into the cell through the patch pipette. It was found that Ca photorelease resulted in a strong Ca-dependent inactivation of the Ca current. This technique was then used to investigate the mechanism of Ca-dependent inactivation. It was found that while both voltage and Ca could inactivate the Ca current, only voltage could immobilize Ca channel gating charge. Thus, voltage- and Ca-dependent inactivation must act through completely independent mechanisms. The hypothesis that Ca inactivates Ca channels by promoting dephosphorylation was tested by studying the effects of isoproterenol. Promoting Ca channel phosphorylation with isoproterenol was found to enhance the Ca current with minimal effects on the gating current. More importantly, isoproterenol could completely prevent Ca-dependent inactivation, even when enough Ca was released to produce an irreversible contracture of the cell. These observations provide strong support for the hypothesis that intracellular Ca ions inactivate Ca channels by promoting dephosphorylation. Interestingly, somewhat different results were observed when nitr-5 was used instead of DM-nitrophen. Ca photorelease from nitr-5 often produced facilitation of the Ca current. This effect was especially prominent when the Ca current was small to begin with. A possible explanation for these results may be that photolysis of nitr-5 does not raise $[Ca]_i$ as much as when DM-nitrophen is used, thus producing less inactivation and allowing a potentiation to be unmasked.

Tu-Pos183

KINETIC PROPERTIES OF L AND N_t- TYPE CA^{2+} CHANNELS AND THEIR ABILITY TO PROMOTE EXOCYTOSIS IN NEUROHYPOPHYSIAL TERMINALS. Martha C. Nowycky, Dept. Anatomy & Neurobiol., Med. Coll. Penn. Philadelphia, PA.

Nerve endings from the mammalian neurohypophysis can be isolated in a form suitable for standard patch clamp techniques. Previously, we showed that individual nerve terminals contain two types of high-treshold Ca^{2+} channels: a classical dihydropyridine (DHP)-sensitive, L-type channel, and a DHP-insensitive channel belonging to the N-family (N_t, Lemos & Nowycky, Neuron 2, 1419, 1989).

Although both channel types are high-threshold, they differ in their kinetics of inactivation. During a maintained depolarization, N_t current inactivates rapidly with a τ_{decay} = ~30 msec, while L-type current shows little decay even with pulses > 1 sec. The N_t type current is completely inactivated at steady-state holding potentials (HPs) =-60 to -50 mV, while the L-type current is partially available even at HP = -10 to 0 mV. Recovery of the N_t type current from inactivation is slow, with $\tau_{recovery}$ = ~400 msec. Trains of simulated action potentials (APs) which mimic physiological activity (2 msec duration, 84 Hz) inactivate the N_t type current within 32-256 pulses, with little effect on L-type.

It has been suggested that the L-type channel supports release of peptide hormones in large dense cored vesicles, while N-type channels are responsible for exocytosis of small classical transmitters. Recently, we applied membrane capacitance measurement techniques to neurohypophysial terminals as an on-line measure of exocytosis (Lim et al., Nature 334, 449, 1990). The contribution of both channels to exocytosis was studied by varying steady-state holding potentials. In terminals in which short duration depolarizations (46 msec) from HP = -90 produced a robust exocytotic response, little response was elicited by the same depolarizing pulses from HP=-50 or -40 mV. However, progressively larger responses could be obtained by increasing the duration of depolarization (between 100 to 260 msec). Thus, both types of channels can contribute Ca^{2+} ions to the release process.

Neurohypophysial peptides are released most efficaciously by characteristic long bursts of APs separated by silent periods. The physiological role of the two high-threshold channels may be to provide a large initial Ca^{2+} influx which promotes facilitation of release (Lim et al.), while at later phases of the burst, the L-type channels continue to supply a smaller, but steady influx of Ca^{2+} ions.

Supported by grants from NINCDS and ICI, Pharmaceuticals.

Tu-Pos184

CAPACITANCE AND CALCIUM CHANGES DURING EXO- AND ENDOCYTOSIS IN SINGLE VERTEBRATE NERVE TERMINALS. M. Lindau* and J. Nordmann†, *Abt. Biophysik, Fb Physik, Freie Universitat, Arnimallee 14, D-1000 Berlin 33, FRG; †Centre de Neurochimie, CNRS, 5 rue Blaise Pascal, F-67048, Strasbourg, France.

We have monitored capacitance changes associated with exocytosis and endocytosis in single isolated nerve terminals using time-resolved patch-clamp capacitance measurements in the 'whole terminal' configuration. These experiments directly demonstrate fusion of large numbers of granules in response to depolarization under voltage clamp. The fine structure of the capacitance changes suggest the fusion of individual granules with the plasma membrane as well as reversible fusion events of large vacuoles. In cells which were heavily stimulated by prolonged depolarization large ON and OFF steps were observed which apparently represent exo- and endocytosis of large vacuoles. During fusion of such a large vacuole a short-lived state with a narrow fusion pore of ~ 1 nS conductance could be observed preceding the long-lived fused state with a wide fusion pore. When terminals are loaded with fura-2 in the 'whole-terminal' patch-clamp configuration, the intracellular calcium concentration increases with a half time of 500-800 ms in response to depolarization to 0.5-1.5 μM and may stay elevated for minutes. The capacitance measurements demonstrate that exocytotic granule fusion may continue for many seconds during prolonged depolarization. Elevation of intracellular calcium in the absence of depolarization also induces granule fusion indicating that the membrane potential change is not essential in stimulating exocytosis. These experiments directly demonstrate the correlations between depolarization, intracellular calcium and exocytosis in nerve terminals. However, the details in the time course of exocytosis and intracellular calcium suggest that the calcium at the plasma membrane, rather than the bulk intracellular calcium, appears to be the essential regulator of exocytotic granule fusion. Supported by DFG-Sfb 312/B6.

Tu-VCR3

CALCIUM CURRENTS IN PARAMECIUM ARE BLOCKED BY ANTIMALARIAL DRUGS

Susan R. Barry*, Juan Bernal#, and Barbara E. Ehrlich#, *Dept. of Phys. Med. & Rehab., Univ. of Michigan, Ann Arbor, MI. and #Div. of Cardiology, Univ. of Connecticut Health Center, Farmington, Ct.

We have studied the effects of the antimalarial drugs, quinacrine, chloroquine, and quinine, on calcium currents in *Paramecium calkinsi*. These compounds are structurally similar to W7, a drug that blocks calcium channels in paramecia.

As an initial test of the effects of the antimalarial drugs on calcium currents, we observed the actions of these drugs on calcium-dependent swimming behavior in parmecia. When the paramecium is placed in a high potassium medium (31mM KCl, 94mM NaCl, 1mM $CaCl_2$, and 10mM MOPS, pH 7.3), the cell swims backward for about 50 seconds. Application of calcium channel blockers, such as W7, reduce the duration of backward swimming. Quinacrine, chloroquine, and quinine all reduced the duration of backward swimming at concentrations ranging from 500nM to 100μM. At a concentration of 10μM, quinacrine inhibited backward swimming by 88%, chloroquine by 37%, and quinine by 29%.

The effects of quinacrine were tested directly on calcium currents using a two microelectrode voltage clamp. The paramecia were bathed in a sodium-free recording medium containing potassium channel blockers (125mM TEA-Cl, 10mM CsCl, 5mM 4-AP, 5mM 2,4-DAP, 15mM $CaCl_2$, 10mM MOPS), impaled with microelectrodes filled with 300mM cesium citrate, and held at a resting potential of-40mV. Depolarizing voltage steps evoked an inward calcium current whose peak amplitude was reduced by 15% in 10μM quinacrine, by 51% in 100μM quinacrine, and by 91% in 1mM quinacrine.

In summary, the effects of quinacrine, chloroquine, and quinine on backward swimming behavior suggest that these drugs inhibit calcium currents. Subsequent voltage clamp studies demonstrate that quinacrine indeed blocks calcium currents. Antimalarial drugs may also reduce calcium currents in other protozoans, including plasmodia, the protozoan parasites that cause malaria. Calcium channel blockade may explain in part the therapeutic effects of these drugs. (Supported by Conservation, Food & Health Found., NIH grant GM39029, and American Heart Assoc.)

Tu-Pos185

PROPERTIES OF A TRANSIENT OUTWARD K^+ CURRENT IN ISOLATED FERRET VENTRICULAR MYOCYTES. D.L. Campbell, Y. Qu., *R.L. Rasmusson, and +H.C. Strauss. Depts. of Pharmacology, +Medicine, and *Biomed. Engineering, Duke Univ. Med. Center, Durham, NC 27710.

The whole cell patch clamp was used to study a transient outward K^+ current I_{to} in single myocytes enzymatically isolated from the right ventricles of 10-16 week old male ferrets. I_{to} is present in Na-free, 5.4 KCl, 2.5 $CaCl_2$ saline (144 NMDG-Cl, 12 μM TTX, 500 μM Cd^{2+}, 22°C), indicating that activation is a voltage-dependent process that does not depend either upon the influx of Na^+ or Ca^{2+}. Activation threshold lies at approx. -10-0 mV, half-activation at approx. +20-+25 mV, and full activation at +40-+50 mV. Activation kinetics are quite rapid, e.g. peaking in approx. 5-10 ms at +50 mV. Whether activation is sigmoidal has not yet been determined. Steady-state inactivation appears to be a conventional voltage-dependent process and is well-fit by a Boltzmann relation ($v_{1/2}$ = -13.5 mV, k = 5.6 mV). Both development of inactivation at potentials below activation threshold (-20-0 mV) and inactivation of macroscopic I_{to} (0-+70 mV) can be well described in most cases by a single exponential, with time constants decreasing with depolarization above -10 mV (e.g +20 mV, 52±13 ms; +70 mV, 36±6 ms). Recovery from inactivation is voltage-dependent and is also well described in most cases by a single exponential, with time constants decreasing with hyperpolarization (e.g. -40 mV, 82±7 ms; -90 mV, 24±8 ms). The overall recovery-inactivation time constant curve is bell-shaped and peaks between -10 and -20 mV. I_{to} can be blocked by 4-AP (K_d approx. 1 mM) and 20 μM quinidine. However, block by 4-AP is complex (see accompanying abstract). Our present data suggests that I_{to} in ferret right ventricular myocytes is composed of a single component. Compared to I_{to} in other mammalian cardiac cell types (e.g. rabbit, rat) I_{to} in ferret right ventricle: (i) has inactivation and activation curves that lie at more depolarized potentials; (ii) recovers much more rapidly. Activation and permeation characteristics of I_{to} are presently being determined.

Tu-Pos186

IDENTIFICATION OF A HYPERPOLARIZATION-ACTIVATED INWARD CURRENT IN YOUNG EMBRYONIC CHICK HEART MYOCYTES. Hiroyasu Satoh and Nicholas Sperelakis, Department of Physiology and Biophysics, University of Cincinnati, College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0576

Young embryonic chick heart cells possess spontaneous activity, whereas cells isolated from 17-day-old embryonic hearts do not. To generate the spontaneous action potentials, a net inward depolarizing current must be produced during the pacemaker potential. Whole-cell voltage-clamp experiments were performed to examine the underlying currents flowing during the pacemaker potential of spontaneously-beating isolated myocytes from embryonic chick ventricle. The holding potential was -30 mV. Long-duration (3 s) hyperpolarizing pulses were applied to -40 to -120 mV, in increments of 10 mV. A marked hyperpolarization-activated inward current (I_f) was produced in 3-day-old cells (-108 ± 6.5 pA, n=5), which was very slowly activated (saturation reached at ca. 3 s). The threshold for activation was about -50 mV. In 17-day-old cells, there was almost no I_f current (-20.9 ± 1.4 pA, n=5). The reduction of I_f paralleled the decrease in spontaneous activity. CsCl (3 mM) blocked I_f completely. In addition, CsCl had a negative chronotropic effect on the spontaneous action potentials: the cycle length went from 935 ± 4 ms (control) to 1851 ± 43 ms (Cs^+) (n=6). These results indicate that a Cs^+-sensitive I_f current exists in young embryonic chick heart cells, and decreases during development. This I_f current may contribute to the electrogenesis of the pacemaker potential. Possible modification of I_f by some autonomic agents is currently under investigation. (Supported by grant HL-31942).

Tu-Pos187

EFFECTS OF DIAZOXIDE AND GLYBURIDE ON ATP-SENSITIVE K^+ CHANNELS FROM HYPERTROPHIED VENTRICULAR MYOCYTES.

Flora Ciampolillo, Deborah E. Tung and John S. Cameron.
Dept. of Biological Sciences, Wellesley College, Wellesley, MA 02181

Hyperpolarizing vasodilators that specifically activate ATP-sensitive K^+-currents ($I_{K(ATP)}$) in smooth muscle have been suggested as an antihypertensive (if potentially arrhythmogenic and/or hyperglycemic) therapy. To date, however, the effects of agents presumed to influence these channels have not been characterized in hypertrophied myocardium. We used standard intracellular and patch-clamp, single-channel recording techniques to study the effects of diazoxide, a vasodilator, and the sulfonylurea glyburide on $I_{K(ATP)}$ in cardiac muscle from control (WKY) and spontaneously hypertensive rats (SHR). Intracellular recordings were obtained from isolated left ventricles; unitary currents were recorded from excised, inside-out membrane patches with symmetrical transmembrane K^+ at 21-23°C. Diazoxide (5-25 μM) caused a decrease in action potential duration (APD) in both WKY and SHR ventricles. This effect was dose-dependent only in control myocardium. In both WKY and SHR, glyburide (5-25 μM) produced dramatic dose-dependent increases in APD approaching 100%. Prior to drug administration, unitary currents in hypertrophied myocytes exhibited a greater mean open-state probability (P_o) upon hyperpolarization than those from control myocytes, although conductance and mean single channel open time were not significantly different. Under patch-clamp, diazoxide (100 μM) increased $I_{K(ATP)}$ in cells from both WKY and SHR. This response reflected an increase in P_o and overall conductance, and was not dependent on intracellular ATP concentration or protein phosphorylation. Glyburide (50 μM) decreased P_o and conductance in both groups. Enhanced activation of $I_{K(ATP)}$ in globally-ischemic, hypertrophied myocardium may provide a protective function by maintaining resting potential or causing local vasodilation. Further drug-induced augmentation of $I_{K(ATP)}$, however, may promote arrhythmias by decreasing APD.

Supported by NIH BRSG 07186-07 and HL-34672.

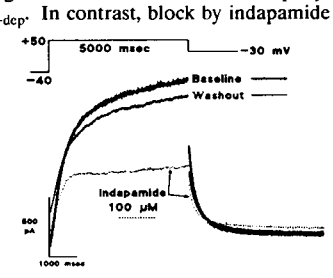
Tu-Pos188

TIME-DEPENDENT BLOCK OF OUTWARD K^+ CURRENT BY THE DIURETIC INDAPAMIDE IN GUINEA PIG MYOCYTES. Jacques Turgeon, Scott Wiggins, Paul Bennett, Dan Roden. Vanderbilt University, Nashville, TN.

Patients developing abnormalities of cardiac repolarization and associated arrhythmias during therapy with K^+ channel blocking agents are often hypokalemic because of concomitant diuretic therapy. Hypokalemia will prolong action potentials; in this study, we tested the complementary hypothesis that diuretics directly inhibit cardiac K^+ current. Chlorthalidone (1 mM) and indapamide (0.01-1 mM) were evaluated in guinea pig ventricular myocytes held at -40 mV and superfused (30°C) with Cd^{2+}-containing solution to block slow inward current. Because time-dependent current ($I_{time-dep}$) in this tissue has multiple components with different activation kinetics, $I_{time-dep}$ was measured during pulses to -10 to +50 mV for 225 msec (short pulses) or 5000 msec (long pulses). While chlorthalidone had no effect, indapamide reversibly blocked $I_{time-dep}$ in a time- and voltage-dependent fashion (100μM, %Δ±SD, n=5-8, all p<0.001 short vs long; K_d for long pulses at +50 mV = 44 μM):

	10 mV	30 mV	50 mV
Short (225 msec)	-3±8%	-17±10%	-23±10%
Long (5000 msec)	-36±16%	-45±10%	-49±10%

Action potential prolonging agents such as E4031 inhibit a rapidly-activating component of $I_{time-dep}$. In contrast, block by indapamide was greatest with long pulses and the indapamide-resistant current activated rapidly. We conclude that indapamide inhibits a slowly-activating component of time-dependent outward current which is not a target for other blockers.



Tu-Pos189

A MAINTAINED OUTWARD K^+ CURRENT IN RAT VENTRICULAR MYOCYTES.

I.D. Dukes & M. Morad, Dept. of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085 and M.D.I.B.L., Salsbury Cove, ME 04672

Recently, we characterized the transient outward K^+ current (I_{to}) in rat ventricular myocytes as being composed of a single component. Activation of I_{to}, however, was often accompanied by activation of a maintained K^+ current, the magnitude of which varied from cell to cell. We now provide evidence that this maintained current represents an independent ionic channel, that differs from both I_{to} and the cardiac delayed rectifier channel (I_K). Cells were dialyzed with an EGTA-containing internal solution (in mM): 120 KCl, 4 NaCl, 5Mg-ATP, 14 EGTA, 10 Hepes pH 7.2, and bathed with a solution containing (in mM): 137 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 Hepes, 10 glucose pH 7.4. Using conditioning pulses from -80 to -10 mV to inactivate I_{to}, test pulses in the voltage range from -20 to +60 mV rapidly (<5ms) activated a maintained K^+ current, $I_{k,m}$. In contrast to I_{to}, which was activated by voltages positive to -40 mV, $I_{k,m}$ was activated at voltages positive to -20 mV. Under steady-state conditions, both currents were completely inactivated at -40 mV, but the time constant of inactivation was 100 ms for I_{to} and about 16s for $I_{k,m}$. Addition of 10 mM 4-AP completely blocked I_{to} without affecting $I_{k,m}$. On the other hand, 10 μM tedisamil blocked both I_{to} and $I_{k,m}$. Both the kinetics of activation of $I_{k,m}$ and the nature of its block by tedisamil resembled the behavior of neuronal I_k (Dukes et al., 1990, J.P.E.T., 254: 560-9,). The kinetics of activation and inactivation of $I_{k,m}$ and the nature of the block by tedisamil were markedly different than the delayed rectifier K^+ channel in guinea pig heart. We conclude, that in addition to I_{to}, rat ventricular myocytes have a non-inactivating K^+ current that shares many of the properties of the neuronal but not cardiac delayed K^+ channel. (Supported by NIH grant HL16152.)

Tu-Pos190

MECHANISM OF ACh ACTION ON i_f IN CANINE PURKINJE STRANDS.

F.Chang, I.S. Cohen, *D. DiFrancesco. SUNY, Stony Brook, NY; *Universita di Milano, Italy

We have recently reported that acetycholine (ACh) can reverse the positive shift of pacemaker current (i_f) activation in canine Purkinje strands while having no direct action of its own (Chang, F. et al., Circ. Res. 66:633-636, 1990). We have now investigated this effect of ACh on the cAMP cascade in more detail. We employed the two microelectrode voltage clamp technique on canine cardiac Purkinje strands of short length (<1.5 mm) and narrow radius (<0.15 mm). In the presence of 4 mM Ba^{2+}, i_f was elicited by applying a 3.5 sec hyperpolarizing pulse from a holding potential of -50 mV. Addition of a membrane permeable analogue of cAMP (8-chlorophenylthio cAMP), 1 mM, increased the amplitude of i_f. This action was not reversed by 1 μM ACh, implying that ACh acts at a step prior to cAMP action. We then looked at the steps controlling intracellular [cAMP]. Inhibiting the phosphodiesterase with 100 μM IBMX increased i_f. This action, however, was reversed by ACh. Finally we investigated whether the action of forskolin, a direct activator of adenylyl cyclase, could be reversed by ACh. 10-20 μM forskolin increased i_f, and ACh at 1 μM at least partially reversed this action of forskolin. These results suggest that ACh acts at a step involved in cAMP production. (See also DiFrancesco et al., J.Physiol. 405:477-491, 1988, for results in sinus node myocytes). Supported by grants HL20558, HL28958, HL43731, and HL35064.

Tu-Pos191

INWARD RECTIFYING K CURRENT IN NORMAL AND HYPERTROPHIED CAT RIGHT VENTRICULAR MYOCYTES.

Ke Zhang, RD Harvey, RL Martin, AL Bassett, RE Ten Eick. Northwestern University, Chicago, IL 60611

It has been reported that the action potential (AP) is prolonged in cardiac hypertrophy, suggesting that membrane current flowing during the AP plateau phase and phase 3, and perhaps the properties of the channels involved with the repolarizing currents may be altered by hypertrophy. Therefore, the kinetics of the inward rectifying K current (I_{K1}) were studied in single right ventricular myocytes, isolated from normal cats and cats with right ventricular hypertrophy (RVH), using the whole-cell-patch clamp technique. The peak amplitude of I_{K1} normalized to the cellular membrane capacitance and the ratio of the peak current to the steady state current were less in RVH than in normal myocytes. The chord conductance of I_{K1} was also less in RVH than in normal myocytes. Studying the effects on I_{K1} of changing external K^+ concentration from 2 mM to 20 mM also suggests that both normalized peak amplitude and chord conductance of I_{K1} were less in RVH than in normal myocytes. The time course of I_{K1} inactivation was fit by a single exponential in both RVH and normal myocytes. The mean slopes of the time constants of inactivation for RVH and normal myocytes were 0.23 ± 0.01 and 0.19 ± 0.03, respectively, indicating that RVH slightly alters the voltage dependence of I_{K1} inactivation. The times to peak current were similar, the voltage dependent activation reaching a peak in 5-15 ms in both RVH and normal myocytes. These findings suggest that hypertrophy can alter I_{K1}, presumably by altering both whole cell conductance and channel gating properties. Whether these changes contribute to the AP prolongation associated with RVH in cat is uncertain.

Tu-Pos192

FURTHER EVIDENCE FOR A NOVEL K^+ CURRENT IN CAT VENTRICULAR MYOCYTES: DIHYDROOUABAIN INHIBITS $I_?$

RL Martin and RE Ten Eick. Department of Pharmacology, Northwestern University, Chicago, IL 60611

We have previously reported an outward transient K^+ current ($I_?$) in single cat ventricular myocytes that develops over time (> 25 min) after obtaining intracellular access using the whole-cell patch voltage clamp technique (Martin et al. 1990, *Biophysical J.* 57:140a). It can be elicited after a hyperpolarizing conditioning step (V_C) from a holding potential of -40 mV by depolarizing test steps (V_t -40 to +60 mV). We have shown $I_?$ is a virtually pure K^+ current which exhibits an unique combination of pharmacological sensitivities and kinetics that set it apart from other previously described K^+ currents found in heart. The possibility that development of $I_?$ is due to Ca^{2+} overload induced over time after gaining intracellular access by our experimental conditions was investigated. If this were the case, addition of dihydroouabain (DHO) should enhance $I_?$. Instead, 100 μM DHO decreased peak amplitude. This action could be explained by: 1) DHO directly blocking the $I_?$ channel or 2) DHO could decrease the current indirectly by its well known effect of inhibiting the Na pump and causing K^+ to accumulate outside the cell. If the slope conductance of $I_?$ were dependent on $[K^+]_o$, this might explain the reduction in peak current seen in the presence of DHO. In contrast, $I_?$ slope conductance was unaffected by changing $[K^+]_o$ from 2.4 to 25.4 mM (n = 4). This data speaks against the idea that the decrease in $I_?$ peak amplitude is solely due to inhibition of the Na pump by DHO. We speculate, that in addition to its well known effect of blocking the Na pump, DHO may have other effects at high concentration. Specifically, DHO may block the ion channel responsible for $I_?$.

Tu-Pos193

INTERACTION OF 4-AP WITH I_{to} IN FERRET VENTRICULAR MYOCYTES. D.L. Campbell, Y. Qu, *R.L. Rasmusson, and H.C. Strauss. Depts. of Pharmacology and *Biomedical Engineering, Duke Univ. Med. Center, Durham, NC, 27710.

4-aminopyridine (4-AP) blocks the transient outward K^+ current I_{to} in numerous preparations. Application of 10 mM 4-AP not only reduces peak I_{to} (+50 mV), but also delays the onset of activation and slows the rate of inactivation (Fig. 1). This results in a crossover of I_{to}. When a double pulse recovery protocol is applied in 10 mM 4-AP I_{to} activated by the second pulse initially increases in a manner consistent with recovery from inactivation, but at longer times becomes progressively reduced (Fig. 2). This is consistent with 4-AP (i) dissociating at depolarized potentials and (ii) associating with a closed state at hyperpolarized potentials.

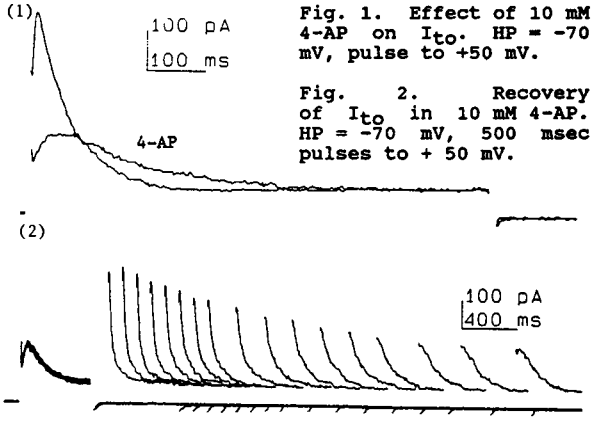


Fig. 1. Effect of 10 mM 4-AP on I_{to}. HP = -70 mV, pulse to +50 mV.

Fig. 2. Recovery of I_{to} in 10 mM 4-AP. HP = -70 mV, 500 msec pulses to + 50 mV.

Tu-Pos194

DYNAMIC CHANGES IN THE INWARD-RECTIFIER (I_{K1}) CURRENT DURING THE CARDIAC ACTION POTENTIAL. A STUDY USING THE ACTION POTENTIAL CLAMP TECHNIQUE. José Ibarra, Gregory E. Morley and Mario Delmar. Department of Pharmacology, SUNY/Health Science Center. Syracuse, NY 13210.

The potassium selective, inward rectifier current, I_{K1}, is responsible for maintaining the resting membrane potential of quiescent ventricular myocytes. However, the contribution of this current to the different phases of the cardiac action potential has not been adequately explored. Single guinea pig ventricular myocytes were used for action potential clamp (APC) experiments. This procedure consists of voltage clamping the cell membrane to its own action potential. Recordings were obtained through a patch pipette in the current clamp configuration and a computer was used to store (via a 12-bit A/D converter) several action potentials. The cell was then switched to single electrode voltage clamp mode and the recorded trace was played back to act as the voltage command for the preparation. In normal Tyrode solution, no net current was recorded except during the pulse (duration, 40 msec; strength 0.5 - 1 nA) that preceded each action potential. However, superfusion with 15 mM cesium (a specific I_{K1} blocker) forced the voltage clamp amplifier to compensate for the absent (Cs- sensitive) component, which yielded an image of the current that would otherwise flow through the blocked I_{K1} channels during the cardiac cycle. The "instantaneous" current-voltage (I-V) relation plotted in this manner shows strong inward going rectification as well as a significant outward component between the resting potential (Vr) and Vr +30 mV. In addition, such a plot reveals that the I_{K1} increases progressively at the subthreshold level of potentials during the current pulse, and that, when just-threshold stimulating pulses are used, I_{K1} reaches the zone of inward-going rectification before the regenerative action potential upstroke ensues. I_{K1} amplitude is strongly reduced during the plateau, but it increases again as the cell repolarizes beyond -20 mV, to act as a major carrier of outward current during the final phase of action potential repolarization. The results also show that the maximal outward current amplitude during repolarization is significantly smaller than during depolarization, thus supporting the hypothesis that I_{K1} rectification is increased by the events which occur during the action potential plateau (Mazzanti M & DeFelice LJ. J Membrane Biol. 116, 41-45, 1990). Our results stress the importance of I_{K1} in the modulation of cell excitability and action potential duration in the ventricular myocyte.

Tu-Pos195

SIMULTANEOUS RECORDING OF ACTION POTENTIAL DURATION AND K_{ATP} CHANNEL ACTIVITY IN CARDIAC VENTRICULAR MYOCYTES. A STUDY USING THE DOUBLE PATCH CLAMP TECHNIQUE. Gregory E. Morley and Mario Delmar. Dept. of Pharmacology. SUNY/Health Science Ctr. Syracuse NY, 13210.

In cardiac myocytes, activation of a particular class of potassium selective channels (K_{ATP}) is known to occur following a drastic fall in the intracellular concentration of ATP ($[ATP]_i$). Recently however, it has been shown that when cell metabolism is inhibited, $[ATP]_i$ remains almost unchanged before irreversible cell damage occurs. These observations have risen the question as to whether K_{ATP} channels are indeed functionally active during the early stages of metabolic blockade, and whether they can be responsible for the observed reduction in action potential duration (APD) following either metabolic inhibition, or prolonged whole cell recordings. To answer these questions, simultaneous recordings of action potentials and membrane channel activity were carried out in single guinea pig ventricular myocytes by means of the double patch clamp technique. Intracellular recordings were obtained with glass pipettes in the whole-cell current clamp configuration. A second, sylgard-coated pipette was filled with normal Tyrode solution and placed on the surface of the myocyte in the cell-attached configuration. Action potentials were elicited by 10 ms current pulses of suprathreshold amplitude (0.8 to 1.2 nA) applied repetitively through the first pipette at a basic cycle length of 1-2 s. At the onset of recording, no active currents were detected with the cell-attached pipette, except for the capacitive surge that was coincident with the action potential upstroke. However, repetitive cell activation (3 experiments) or addition of 0.1 mM of 2-4- di-nitro-phenol (DNP) to the superfusate (3 experiments) led to shortening of action potential duration and to a concomitant appearance of channel activity. The recorded channel currents were outward for the entire voltage range of the action potential, did not show any time dependence, and their amplitude increased linearly with cell depolarization. A channel conductance of 32 pS was measured from an experiment in which single channel activity was clearly discernible. These characteristics are strongly suggestive of K_{ATP} channel activity. Our results directly demonstrate that the progressive abbreviation of APD that occurs either spontaneously during whole-cell recordings, or following metabolic inhibition, results from activation of K_{ATP} channels. It is highly possible that these channels are responsible for the APD abbreviation during anoxia, and that they are regulated by another mechanism besides the cytoplasmic concentration of nucleotides.

Tu-Pos196

DENSITY OF ATP-SENSITIVE K-CHANNELS INFLUENCES THE RESPONSE OF NEONATAL RAT MYOCYTES TO METABOLIC INHIBITION. G. Haddad*, O.F. Schanne and E. Ruiz-Petrich, Dept of Physiol. and Biophysics, Univ. of Sherbrooke, Qué, Canada J1H 5N4.

Glibenclamide was used in voltage and current clamp experiments on neonatal rat ventricular cells to assess whether sparsity of ATP-sensitive K channels underlies the low sensitivity of these cells to metabolic inhibition. The patch clamp technique in whole cell configuration was used with 2 mM Co in the extracellular medium and no ATP in the pipette. In 59 cells we obtained the following ($\bar{x}$ ± SEM): Rinp, 506.86 ± 37.56 MΩ, surface area, 1594 ±6.9 μm^2, Rm, 7.69 ± 0.57 $K\Omega cm^2$. 2-Deoxyglucose (2-DG, 5 mM) shortened APD-95 by 43% with 3 mV hyperpolarization. In 7 out of 12 cells, glibenclamide (100 µM) reduced the resting potential by 6 mV and reversed AP shortening by lengthening phase 3 of the action potential. In voltage clamp experiments, steps of 10 mV and 300 ms were applied from a holding potential of -80 mV in the voltage range of -140 to + 40 mV. After 20 min of exposure to 5 mM 2DG the conductance of the steady state outward current increased from 53.3 to 213 $\mu S/cm^2$; the amplitude of the initial inward current did not change but the negative slope due to I_{K1} relaxation at negative potentials was reduced. Glibenclamide eliminated the outward current induced by 2DG and reduced its conductance to 50 $\mu S/cm^2$. The inward current decreased beyond control levels (42.5 compared to 132.5 $\mu S/cm^2$), with elimination of the I_{K1} relaxation. These observations correlate with the depolarization and the increase in APD-95 beyond control in the current clamp experiments. We can conclude that the 2-DG induced current was due to opening of K-ATP channels whose density is estimated at 0.1 μm^{-2}, 100 times lower than in the adult rat. This suggests that in the glibenclamide-insensitive cells these channels were not yet expressed.

Supported by MRC Canada and QHF. *Supported by CIDA.

Tu-Pos197

THE ATP-SENSITIVE K CHANNELS ARE NOT THE WHOLE STORY. F. deLorenzi, D. Chartier and E. Ruiz-Petrich. (Intr. by M.D. Payet), Dept of Physiology and Biophysics, Univ. of Sherbrooke, Sherbrooke, Q.C. Canada J1H 5N4.

The nature of the K current(s) underlying the action potential (AP) shortening in metabolically depressed myocardium remains controversial. Extensive experimental evidence points to a predominant role of ATP-sensitive K channels but other mechanisms or modulating effects are necessary to explain: a) different time course of AP shortening and ATP depletion in perfused hearts, b) the specific blocker glibenclamide (GLI) only partly reverses AP shortening, and c) long delay before the onset of GLI action. We studied the effects of hypoxia and cyanide on propagated and membrane action potentials and steady state currents in perfused hearts and myocytes from rabbits. The presence of a gK-ATP and its sensitivity to GLI was assessed by previous exposure to lemakalim (LEM) or pinacidil (PIN). The APs of isolated myocytes and their response to changes in stimulation frequency between 0.1 and 3 Hz were comparable to those obtained in whole hearts. The control steady state current showed the properties of IK1 and the inward rectification was abolished by 50 μM Ba^{2+}. The membrane resistance at ± 10 mV from the resting potential (5.8 ± 1.2 and 4.3 ± 1.1 KΩ cm^2) increased under Ba^{2+} (23.7 ± 5.6 and 23.0 ± 5.9 KΩ cm^2). NaCN (0.4 to 2 mM) produced the classic increase in outward current. Exposure to PIN (50 μM) or LEM (50 μM) depressed propagated APs, abolished membrane APs and the net inward current, and triggered a strong outward current. These effects were reversed by GLI (10-30 μM). Addition of cyanide or hypoxia under these conditions still elicited an increase of outward current on depolarization and an AP shortening. The kinetics of the CN-induced current ressembled that of a delayed outward rectifier. These results favor the view that IK-ATP is not the only current system involved in AP shortening during myocardial metabolic depression.

Supported by MRC Canada and FMCQ.

Tu-Pos198

Mg^{2+} REGULATION OF BASAL AND β-ADRENOCEPTOR STIMULATED DELAYED RECTIFIER K^+ CURRENT IN BULLFROG CARDIAC ATRIAL CELLS. Isabelle Duchatelle-Gourdon, Armando A. Lagrutta & H. Criss Hartzell. Department of Anatomy and Cell Biology. Emory University School of Medicine. Atlanta, GA 30322.

Initial variations in the amplitude of whole-cell delayed rectifier potassium current after patch break were correlated with the free $[Mg^{2+}]_i$ present in the pipette. Run-down of I_K appeared with $[Mg^{2+}]_i$ > 1 mM and run-up with $[Mg^{2+}]_i$ < 1 mM, within a range of 0.1 to 3 mM. Basal free $[Mg^{2+}]_i$, indicated by I_K amplitude prior to run-down or run-up, was estimated to be between 0.8 and 1 mM. The amplitude of the activation and instantaneous IV curves was also increased in low $[Mg^{2+}]_i$ and decreased in high $[Mg^{2+}]_i$. However, neither I_K voltage dependence nor reversal potential were affected, ruling out a voltage-dependent block similar to the one reported for the inward rectifier K^+ channel family or an intracellular charge screening effect. The rate of activation of I_K at +40 mV was increased in lower $[Mg^{2+}]_i$ without changes in the rate of deactivation at -50 mV.

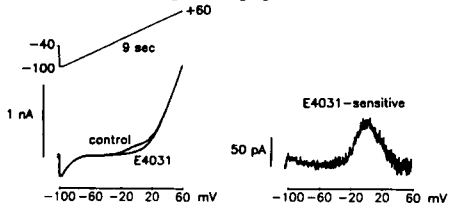
I_K amplitude was increased by β-adrenergic agents like isoproterenol (ISO) by about 2.5 pA/pF, regardless of $[Mg^{2+}]_i$ and whether I_K had previously run down or run up. This could mean that either Mg^{2+} and ISO act on 2 different sites on the same channel or that 2 different populations of channels are involved. In addition, the reversibility of ISO and cAMP on I_K, but *not* on I_{Ca}, was partially inhibited for $[Mg^{2+}]_i$ < 1 mM. At this point, blocking the protein kinase A with 200 μM Rp-cAMP did not restore the reversibility of phosphorylation with PKA on I_K. It is reasonable to assume that the reversibility of the effects of ISO and cAMP reflect the dephosphorylation of the channels. Low $[Mg^{2+}]_i$ levels could turn the K^+ channels into poor substrates for the phosphatases or reduce the activity of Mg^{2+}-dependent phosphatases involved in the dephosphorylation of K^+ channels only.

Supported by NIH grants HL27385 and HL21195 and by the Fondation Simone et Cino del Duca.

Tu-Pos199

I_K IS COMPRISED OF TWO CURRENTS IN GUINEA PIG ATRIAL CELLS. M.C. Sanguinetti and N.K. Jurkiewicz, Merck Sharp & Dohme Research Labs, West Point, PA 19486.

The delayed rectifier K^+ current (I_K) was studied in isolated guinea pig atrial myocytes using the whole cell voltage clamp technique. Similar to previous findings in ventricular cells (J. Gen. Physiol. 96:195, 1990), I_K of atrial cells is the composite of two distinct components: I_{Kr}, a rapidly activating current that exhibits strong inward rectification and I_{Ks}, a slowly activating current with only modest rectification. I_{Kr} was defined by its sensitivity to block by Co^{2+} and the Class III antiarrhythmic agent, E-4031. I_{Kr} underlies the prominent outward "hump" (between -30 and +40 mV) in the steady-state current-voltage relationship determined in voltage ramps (see figure). Activation of I_{Kr} was not dependent upon transient changes in intracellular $[Ca^{2+}]$. Block of Ca^{2+} current by nisoldipine or nitrendipine did not prevent activation of I_{Kr}. Peak I_{Kr} was not decreased in cells when intracellular Ca^{2+} was strongly buffered with BAPTA. The activation curve for I_{Kr} in atrial cells had a threshold of -40 mV, a half-point of -19 mV and a slope factor of 5.3 mV. The activation curve for I_{Ks} had a half-point of +24 mV and a slope factor of 15.7 mV. The peak tail currents of fully activated I_{Ks} (21.1 pA/pF) and I_{Kr} (2.53 pA/pF) are about two-fold greater than that previously measured in guinea pig ventricular cells. This difference may partly explain why action potentials of atrial cells are shorter than those of ventricular cells in guinea pig hearts.

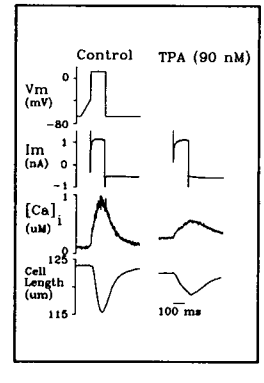


Tu-Pos200

ACTIVATION OF PROTEIN KINASE C BY PHORBOL ESTERS ALTERS THE KINETICS OF THE INTRACELLULAR CALCIUM ($[Ca^{2+}]_i$) TRANSIENT AND THE TWITCH IN SINGLE VENTRICULAR MYOCYTES FROM ADULT RAT HEART. **M.S. Kirby, T.B. Rogers & W.J. Lederer.** University of Maryland, Baltimore, MD 21201.

Activation of protein kinase C (PKC) in heart muscle has diverse electrical and mechanical actions that we have studied in single rat heart muscle cells. We examined the actions of TPA using a whole-cell patch-clamp method while simultaneously measuring intracellular calcium, $[Ca^{2+}]_i$ using indo-1 fluorescence, and cell length (using video methods). Cells were superfused with physiological saline containing 2 mM Ca and TTX (30 μM) at 35°C, pH 7.4. The patch pipette contained (mM): 140 KCl, 1 $MgCl_2$, 9 K_2ATP, 4 Na_2CP, 10 HEPES and 0.07 K_2Indo, pH 7.15 at 35°C.

The figure below shows the voltage protocol used (repeated at 0.5 Hz) and the action of TPA on membrane current (I_m), on $[Ca^{2+}]_i$ and on cell length. 7 minutes exposure leads to a decrease in I_{Ca} (Cd-sensitive current, not shown), $[Ca^{2+}]_i$ and the twitch. Additionally, the time-to-peak $[Ca^{2+}]_i$ is delayed and the $t_{1/2}$ of $[Ca^{2+}]_i$ relaxation on repolarization is increased (from 106 to 248 ms). Parallel changes in twitch shortening are seen. These findings not only support the decrease in peak $[Ca^{2+}]_i$ during the twitch reported by Cappogrossi *et al.*, Circ. Res. 66, 1143-1155) in electrically stimulated action potential experiments (i.e. no voltage-clamp) but also point to regulation of $[Ca^{2+}]_i$ not apparent in these experiments. The slowed kinetics of the $[Ca^{2+}]_i$ transient and twitch may arise from PKC-dependent alteration of sarcoplasmic reticulum function recently described (Rogers *et al.*, J.Biol.Chem. 265, 4302-4308).



Supported by the N.I.H. and the Maryland Affiliate of the American Heart Association.

Tu-Pos201

ACTIVATION OF A HEART CHLORIDE CHANNEL DURING STIMULATION OF PROTEIN KINASE C. Kenneth B. Walsh. Department of Pharmacology, University of South Carolina, Columbia, 29208.

A β-adrenergic-sensitive Cl^- current has recently been identified in cardiac ventricular myocytes (Harvey and Hume, Science 244, 983 (1989); Bahinski et al., Nature 340, 718 (1989)). The purpose of this study was to determine if heart ventricular cells contain a protein kinase C (PKC)-sensitive Cl^- current and to investigate the effect that chiral derivatives of the monocarboxylic acid 2-(4-chlorophenoxy)propionic acid (CPP) have on these channels. Ion currents were recorded from guinea pig ventricular myocytes using the whole-cell arrangement of the patch clamp technique. In the presence of appropriate external solutions and drugs to reduce contamination from Na^+, Ca^{2+} and K^+ currents, application of the phorbol esters phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate, to stimulate 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 PKC-sensitive current in manner expected for a Cl^--selective ion channel. Both the PKC-sensitive current and the Cl^- current activated by the membrane soluble cAMP analog 8-chlorphenylthio cAMP were reversibly inhibited by the S(-) and R(+) enatiomers of CPP. Thus, heart ventricular cells contain Cl^- channels which can be activated through cAMP-dependent and independent pathways. The ability of purified protein kinase C to activate single Cl^- channels in excised patches is currently under study.

Tu-Pos202

EFFECTS OF PHORBOL ESTER ON THE RELATIONSHIPS BETWEEN INTRACELLULAR pH, $[Ca^{++}]$ AND CONTRACTION IN ISOLATED RAT CARDIAC MYOCYTES.

Kenneth T. MacLeod and Sian E. Harding. *Department of Cardiac Medicine, National Heart & Lung Institute, Dovehouse Street, London, SW3 6LY, U.K.*

In many tissues activation of protein kinase C (PKC) has been shown to stimulate Na^+/H^+ exchange and mobilise Ca^{++}, but its action in cardiac muscle is unclear. We have examined the effects of phorbol esters, some of which activate PKC, on contraction, intracellular pH (pH_i) and intracellular [free Ca^{++}] ($[Ca^{++}]_i$) in isolated, Ca^{++}-tolerant rat ventricular myocytes at 32°C using the fluorescent indicators BCECF and Fura-2. Application of 10^{-7} - 10^{-6}M phorbol 12-myristate 13-acetate (PMA), which stimulates PKC, caused a positive inotropic effect. 4-phorbol 12,13-didecanoate (PDD), which does not activate PKC, did not alter myocyte contractility. Two mechanisms which may bring about the positive inotropy are (1) PKC activation of Na^+/H^+ exchange so causing an intracellular alkalinization and/or (2) an increase in peak systolic $[Ca^{++}]_i$.

While PMA had no clear effect upon steady-state pH_i our experiments show that the exchanger can indeed be stimulated by activated PKC. Recovery of pH_i from an acidosis (imposed by addition then subsequent removal of 10mM NH_4Cl) was more rapid by 15.1 ± 6.9% (mean ± SEM; n = 13; $p < 0.05$, paired t-test) in the presence of PMA than in control. The recovery of shortening from an acidosis was always more rapid (by 59.1 ± 10.6% ; n = 5; $p < 0.05$, paired t-test) in the presence of PMA. Throughout the range of pH_i tested, the plot of logarithmic cell shortening is linear having a slope of 1.99 in control and 1.13 in the presence of PMA. The presence of PMA shifts the relationship upwards such that at any one pH_i, cell shortening is greater. PMA also increased peak systolic $[Ca^{++}]$ by 21 ± 4% (mean ± SEM; n = 5) and it is this increase which may be responsible for the positive inotropic effect though changes in contractile protein sensitivity cannot be ruled out.

Supported by the British Heart Foundation and Medical Research Council.

Tu-Pos203

ALPHA$_1$-ADRENOCEPTOR STIMULATION PRODUCES A POSITIVE INOTROPIC EFFECT WHICH OCCURS WITH A DECREASE IN THE CA^{2+} TRANSIENT AND THE ACTION POTENTIAL DURATION IN GUINEA PIG VENTRICLE. ROBERT T. DIRKSEN, RU-CHI SHIEH, DANIEL J. WILLIFORD, AND SHEY-SHING SHEU. Department of Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642.

Alpha$_1$-adrenoceptors have been demonstrated to produce a time- and dose-dependent positive inotropic effect and shortening of the action potential duration (APD) in guinea pig ventricle. The effects of a maximal concentration (100 μM) of the alpha$_1$-adrenoceptor agonist methoxamine (MTX) on specific ionic currents that contribute to the ventricular AP were investigated in isolated myocytes using the whole-cell patch clamp technique. Propranolol (1 μM) was present in all experiments to block any beta-adrenoceptor-mediated effects. MTX produced no significant effect on the L-type Ca^{2+} current. Moreover, MTX significantly decreased the maximal tail current amplitude of the fully activated delayed rectifier potassium current elicited by depolarizing pulses from -30 mV to +30 mV for 2 seconds. Tail current envelope experiments of this K^+ current revealed MTX produced no significant change in the fraction of this maximal current elicited by briefer pulses. In a chloride-free extracellular solution MTX effects on the APD in papillary muscles were similar to control. These results eliminate the involvement of the L-type Ca^{2+}, delayed rectifier potassium, and outward chloride currents in the MTX-induced shortening of the APD.

The effects of MTX (100 μM) on action potentials and fura-2 Ca^{2+} transients in single right ventricular myocytes were simultaneously studied using high resistance microelectrodes. MTX decreased the APD (14.7 ± 4.6%) and the peak Ca^{2+} transient (15.3 ± 6.8%) with the same time course, suggesting that the two effects may be related. These results demonstrate for the first time a positive inotropic agent that decreases the Ca^{2+} transient. Furthermore, these results are consistent with an alpha$_1$-adrenoceptor-mediated increase in sensitivity of the myofilaments to Ca^{2+}, as has been previously suggested.

Tu-Pos204

PHENYLEPHRINE INCREASES CYTOSOLIC FREE CALCIUM CONCENTRATION IN ATRIAL HEART CELLS FROM RATS

U. Jahnel*, H. Nawrath*, R.-C. Shieh, V. K. Sharma, D. J. Williford, S.-S. Sheu
From the *Department of Pharmacology, University of Mainz, Federal Republic of Germany and the Department of Pharmacology, University of Rochester, School of Medicine and Dentistry, Rochester, New York.

The effects of phenylephrine (PE) and isoprenaline (ISO) on Ca^{2+} current (I_{Ca}) and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were compared in isolated atrial and ventricular myocytes from rats. PE did not significantly affect magnitude or time course of I_{Ca} in either tissue, whereas large increases of peak I_{Ca} were observed in response to ISO. $[Ca^{2+}]_i$ was evaluated fluorometrically in myocytes previously loaded with the fluorescent dye fura-2. In atrial cells, PE evoked an increase in diastolic $[Ca^{2+}]_i$ during activity and, ocassionally, also at rest; peak $[Ca^{2+}]_i$ transients were either unchanged or slightly increased. When diastolic $[Ca^{2+}]_i$ was increased to a greater extent, $[Ca^{2+}]_i$ transients were decreased or abolished. PE also caused a concentration-dependent decrease in resting potential (RP) and a prolongation of the action potential. Simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential showed that both increase in diastolic $[Ca^{2+}]_i$ and decrease in systolic $[Ca^{2+}]_i$ transients were associated with the decrease in RP. To compensate for the change in RP produced by PE, hyperpolarizing voltage clamp pulses of 200 ms, preceeding the AP, were applied. During the clamp, the PE-induced increase in $[Ca^{2+}]_i$ was completely eliminated and $[Ca^{2+}]_i$ transients were slightly increased when the AP started from the original membrane potential. In ventricular cells PE did not change either RP or diastolic $[Ca^{2+}]_i$; $[Ca^{2+}]_i$ transients were slightly increased. ISO caused large increases in $[Ca^{2+}]_i$ transients in atrial and ventricular cells, whereas diastolic $[Ca^{2+}]_i$ remained virtually unchanged, except at high concentrations of the drug with concomitant changes in membrane potential. The results suggest different mechanisms whereby PE and ISO increase $[Ca^{2+}]_i$ in rat atrium. The positive inotropic effect due to the stimulation of β-adrenoceptors is commonly ascribed to an initial Ca^{2+} influx through voltage-dependent Ca^{2+} channels, triggering the release of Ca^{2+} from intracellular stores and, subsequently, leading to a transient increase of cytosolic $[Ca^{2+}]_i$. In contrast, the positive inotropic effect of PE is obviously not related to any changes in I_{Ca}. The increase in diastolic $[Ca^{2+}]_i$ in response to PE is probably a consequence of the changes in RP, possibly involving the activation of the Na^+-Ca^{2+} exchange mechanism.

Tu-Pos205

MODULATION OF INTRACELLULAR Ca^{2+} TRANSIENTS AND REPOLARIZATION OF ACTION POTENTIALS BY α_1 ADRENERGIC STIMULATION IN THE PRESENCE OF β_1 AGONISTS IN CANINE CARDIAC VENTRICULAR MYOCYTES.

Raed Sweidan, Tamas Banyasz, Robert D. Fugate, Bela Szabo and Ralph Lazzara, University of Oklahoma Health Sciences Center, Oklahoma City, OK

β_1 stimulation with isoproterenol (ISO) induces delayed afterdepolarizations (DAD) with triggered action potentials (TAP). Myocytes loaded with fura 2AM were stimulated and DAD as well as TAP were recorded via 30-60 Mohm microelectrodes with an Axoclamp 2A. Ca^{2+} transients (CAT) in the cytosol associated with stimulated action potentials (AP) were measured as changes in the ratios of fluorescence intensity of fura 2 at 510 nm when exciting at 340 and 380 nm chopped at 0.3 kHz, using an SLM DMX-1000 interfaced to a Nikon Diaphot microscope. [ISO]$\geq$ 1.0 nM increased the amplitudes and reduced the relaxation time of CAT during AP, spontaneous CAT (SCAT) occurred with DAD and TAP which increased with pacing rate. Methoxamine (MET) is known to activate α_1 receptors and we found that it also prolongs the relaxation time of CAT during AP with the simultaneous generation of early after-depolarizations (EAD) which were further enhanced by reductions in pacing rates. MET inhibited DAD at all pacing rates. We conclude that α_1 modulates β_1 stimulation of CAT by prolonging the relaxation of CAT and preventing the generation of DAD and TAP. However exaggerated α_1 effects prolong CAT which induce EAD with TAP. The observed effect of α_1 and β_1 may reflect alterations in kinetics of sequestration of cytosolic Ca^{2+} at the end of the electro-mechanical relaxation of the myocyte.

Tu-Pos206

ALLOSTERIC KINETICS OF CROSS-LINKED HEMOGLOBIN

Mingdi Zhao*, Jie Jiang*, Frank A. Ferrone*, Mark E. Andracki†, Scott A. Fowler† and Joseph A. Walder†

*Department of Physics & Atmospheric Science, *Drexel University*, Philadelphia PA 19104 and †Department of Biochemistry, *University of Iowa*, Iowa City, Iowa 52242

We have used the method of modulated excitation to measure the allosteric kinetics of human hemoglobin covalently cross linked at β 99. By partial oxidation followed by isoelectric focussing, it is possible to prepare triply ferric species which form the majority of the sample. In typical modulation experiments, great care must be taken to excite only a single heme per tetramer, and this is accomplished by exciting less that 1% of the sample. The triferric derivatives allow an order of magnitude improvement in the signal size, as well as permitting intriguing glimpses into the allosteric behavior of the protein. Both oxygen and carbon monoxide photolysis can be studied. There is little allosteric signal observed for O_2, while a clear signal is seen for CO. With both ligands, an unknown but prominent spectral feature occurs in the modulated spectrum at the peak of the ferric subunits (following the static spectra as ferric ligands are changed) , which we tentatively ascribe to a photoreduction process.

Tu-Pos207

ALLOSTERIC SPECTRA GENERATED BY AUTOMATED OXYGEN BINDING MEASUREMENTS

Michael Greene and Frank A. Ferrone,

Biomedical Engineering and Science Institute, and Department of Physics & Atmospheric Science, *Drexel University*, Philadelphia PA 19104

In order to obtain allosteric signature spectra for use with modulated excitation studies of hemoglobin, we have constructed a novel automated thin layer oxygenation appartus. The oxygen chamber uses an oxygen permeable membrane and is based on the design of Gill (cf. *Methods in Enzymology*, **76** 431 (1981)). For control of oxygen pressure we employ precision mass flow controllers, and gas mixtures to allow computer selection of the fraction of oxygen between 10^{-4} and 1. This approach allows data collection to be optimized for regions of maximal spectral change. Spectra are collected by a Hewlett Packard 8452 Diode Array spectrophotometer, and analyzed by singular value decomposition. Full spectral analysis also permits such problems as drifts and sample instabilitiy to be rapidly identified and corrected.

Tu-Pos208

MONOMER DIFFUSION AND POLYMER ALIGNMENT IN DOMAINS OF SICKLE HEMOGLOBIN

Michael R. Cho* and Frank A. Ferrone,

Department of Physics & Atmospheric Science, *Drexel University*, Philadelphia PA 19104

Polymerization of sickle hemoglobin forms arrays called polymer domains, which grow with circular symmetry and subsequently produce aligned polymers. Immobilization of monomers in polymers leads to diffusion into the domain and an increase in total concentration. The concentration finally attained is between 42 and 50 g/dl, with little or no dependence on final temperature, initial concentration, or temperature of formation. The approach to a final value occurs with a slow, non exponential progress curve characteristic of diffusion, and with half times that scatter about 50 s (for 22 μm domains) with little sensitivity to temperature or concentration. The half time decreases upon decreasing the domain size (5 fold decrease for a factor of 2.2 in size), or upon production of multiple domains. During the increase in concentration, the gradient within the domain is very small, suggesting rapid diffusion, which agrees with calculations based on known diffusion rates. Alignment of polymers, measured as linear dichroism, occurs in parallel with monomer diffusion, with the same progress curves and characteristic times. These findings may be rationalized by assuming that the monomer diffusion into the domain is rate limited by some polymer reorganization process which is also responsible for the observed alignment transition.

* present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

Tu-Pos209

THEORY OF LIGHT SCATTERING AND ABSORPTION OF SICKLE HEMOGLOBIN DOMAINS VERSUS POLYMERS.

J. R. Wheeler and Marilyn F. Bishop, Department of Physics, Virginia Commonwealth University. 1020 West Main Street, Richmond, VA 23284-2000.

We have calculated the light scattering and absorption of a solution of sickle hemoglobin (HbS) polymers, separately accounting for the contributions from domains versus individual polymers. For simplicity in these calculations, we assume that the domains and the polymers are well separated from one another and are small compared with the wavelength of incident light. For the spherulite calculation, we have used a model of an inside anisotropic and an outside isotropic uniform effective medium dielectric. The effective medium represents, inside the domain, the average properties of the polymers, monomers, and solution, and outside, the average properties of a medium composed of monomers and solution. Polymers are represented by finite ellipsoids with the same length to diameter ratio as HbS polymers, and hemoglobin molecules (monomers) and solution "particles" are represented by spheres. When absorption is included, the dielectric constant inside individual polymers and monomers is anisotropic, and an enhanced anisotropy is obtained when polymers are aligned due to the elongated shapes of the particles. The light scattering from domains depends strongly on the alignment of polymers within a domain, especially in regions where the absorption is small. In regions of large absorption, light scattering from domains increases dramatically over the non-absorptive regions and is sizable even if the polymers within a domain are not aligned.

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Tu-Pos210

PHASE TRANSITION IN HIGH IONIC STRENGTH SOLUTIONS OF DEOXY SICKLE CELL HEMOGLOBIN. Marion Patterson, Erick Y. Nana, Kesia Alexander and Muriel S. Prouty, Chemistry Department, University of the District of Columbia, Washington, D.C.

The distinctive gel form of the condensed phase of deoxy sickle cell hemoglobin (HbS) occurs in the red blood cell and in deoxy HbS solutions, in appropriate buffers when pH and ionic strength are not too far from physiological. Such buffers include dilute phosphate and bistris at pH close to 7. Prouty et al showed previously that in 0.15M phosphate buffer at pH 7.1, the removal of solvent from dilute deoxy HbS solutions by osmotic stress brings about the solution-to-gel phase transition at the same concentration (C_{sat}) as measured by ultracentrifugation assay. The transition under osmotic stress is accompanied, however, by a significant loss of solvent--resulting in the dense gels more characteristic of older RBC and ISC. We have now studied this phase transition in high ionic strength phosphate buffers, and in 0.15M phosphate with ionic strength increased by added chloride or sulfate salts. Solubility decreases with increasing ionic strength as expected, and where the condensed phase is a gel, a three-zoned phase diagram is seen: solution, the sharp transition accompanying polymerization, and then gradual increase in concentration from packing and alignment of fibers. At ionic strengths above which Asakura has described an "aggregate" rather than a gel, we find the osmotic stress phase diagram characteristic of a solution-to-crystalline precipitate transition. Distinct crystals can be seen in osmotic sacs from these experiments. We had previously seen this transition in lysozyme. (Supported by NIH AREA, MBRS and MARC grants.)

Tu-Pos211

ERYTHROCYTE MEMBRANES AND SICKLE CELL HEMOGLOBIN POLYMER GROWTH. P. J. Baxter and H. Mizukami. Division of Regulatory Biology and Biophysics, Wayne State University, Detroit, Michigan, 48202.

Extensive studies have been performed on the formation of sickle cell hemoglobin (HbS) polymers in solution. However, the intrinsic environment of the red cell millieu and the interaction of the hemoglobin and the inner surface of the membrane cannot be ignored. The possibility that the cell membrane may be interacting with the polymerizing HbS has remained unanswered. In this investigation, transmission electron microsopy and polarization light microscopy were used to examine the formation of HbS nuclei formation at the cell membrane and subsequent polymer growth.

Inside-out vesicles (IOV's) were prepared from the erythrocytes of patients with sickle cell disease by a modified method of Steck[1]. Sickle cell hemoglobin was chromatographically pure and was used at a concentration of at least 20%. The IOV's and HbS were oxygenated before mixing, and then deoxygenated in a nitrogen filled glove box. Complete deoxygenation was insured by adding a stoichiometric amount of sodium dithionite. The samples were fixed with glutaraldehyde-formaldehyde, post-fixed with OsO_4, and stained *en bloc* with uranyl acetate. The samples were then embedded and sectioned for viewing.

Our observations suggest the the inner surface of the erythrocyte may be serving as a template for polymer nucleation. (Supported in part by a grant from NIH HL 16008)

[1] Steck, T. L. (1974) Preparation of Impermeable Inside-Out and Right-Side-Out Vesicles from Erythrocyte Membranes, in: *Methods in Membrane Biology, Vol. 2* E.D. Korn (ed.), Plenum Press, NY, pp 245-281.

Tu-Pos212

HOMOGENEOUS NUCLEATION RATE DETERMINES THE STRUCTURE OF HEMOGLOBIN S GELS AND THE SHAPE OF SICKLED CELLS. Garrott W. Christoph, James Hofrichter, and William A. Eaton. Laboratory of Chemical Physics, NIDDK, NIH, Bethesda Maryland 20892.

Gels of Hb S are organized into domains of polymers that cause "sickling". If a single homogeneous nucleation event triggers the formation of an entire domain, then the double nucleation mechanism for Hb S polymerization (Ferrone, Hofrichter, and Eaton, J. Mol. Biol. 183, 611 (1985)) may be used to explain the enormous variation in the structure of gels and in the morphology of cells. More specifically, the model predicts that (i) the equilibrium concentration of domains depends approximately inversely on the delay time, (ii) the distribution of domain volumes is exponential, and (iii) the shape of the deoxygenated sickle cell depends on the rate of polymerization. To test these ideas we have determined the distribution of domain sizes on deoxyHb S gels formed with known delay times. Kinetic progress curves following a temperature jump were measured on 10-20 μm layers using linear birefringence measurements with a polarizing microscope to monitor gel formation. Domain diameters were measured from video images obtained between crossed linear polarizers on the resulting gel. Like the delay time, the concentration of domains is proportional to about the 30th power of the Hb S concentration. As a result, the concentration of domains is inversely proportional to the 0.9 power of the delay time, in agreement with the prediction of the double nucleation mechanism. Also, the distribution of domain volumes rises sharply and decays with a broad exponential tail, as predicted by the model. Finally, experiments on sickle cells show that rapid deoxygenation, resulting in rapid intracellular gel formation, produces undistorted cells with multiple domains, while slow deoxygenation produces a variety of highly distorted ("sickled") cells with one or few domains.

Tu-Pos213

HEMOGLOBIN CRYSTALS BIND OXYGEN NON-COOPERATIVELY WITH NO BOHR EFFECT. Andrea Mozzarelli, Claudio Rivetti, Gian Luigi Rossi, Eric R. Henry, and William A. Eaton (Intr. by David R. Davies). Institute of Biochemical Sciences, University of Parma, 43100 Parma, Italy, and Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, Maryland.

Hemoglobin remains the paradigm for understanding the cooperative binding behavior of proteins because a surprisingly simple model can explain the major features of a vast array of structural, spectroscopic, equilibrium, and kinetic data. According to this MWC-PSK model (Monod, Wyman, and Changeux, J. Mol. Biol. 12, 88 (1965); Perutz, Nature 228, 726 (1970); Szabo and Karplus, J. Mol. Biol. 72, 163 (1972)) salt bridges stabilize the T quaternary structure relative to R, and act as the constraints that cause the low affinity of the T state; breakage of the salt bridges, either by oxygen binding to the T state or by the quaternary conformational change from T to R, releases the protons that are responsible for the Bohr effect. The model is primarily based on a comparison of X-ray structural studies on crystals with functional studies on solutions. To more directly test the model we have used a microspectrophotometer to measure oxygen binding curves of single crystals of human hemoglobin grown from polyethylene glycol. These crystals remain in the T quaternary structure with intact salt bridges upon oxygenation at pH 7.0 (Liddington *et al.*, Nature 331, 725 (1988)). As predicted by the model, there is little or no cooperativity in the oxygen binding. Furthermore, the affinity is almost independent of pH between 6.0 and 8.5. This absence of a Bohr effect in the T quaternary structure is also predicted by the model, provided that further X-ray crystallographic studies show that the salt bridges remain intact at alkaline pH as well. Although the α hemes bind oxygen with slightly higher affinity than the β hemes, the inequivalence is much less than that suggested by the X-ray results.

Tu-Pos214

R-LIKE TRANSITION STATE EXPLAINS RELATION BETWEEN QUATERNARY RATES AND EQUILIBRIA IN HEMOGLOBIN. William A. Eaton, Eric R. Henry, and James Hofrichter (Intr. by Gary Felsenfeld), Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, Maryland 20892.

In a recent study on trout Hb (Hofrichter et al., submitted to Biochemistry) the activation enthalpies and entropies for the T_0 to R_0 quaternary conformational change were found to be much more similar to the equilibrium enthalpies and entropies than the corresponding values for the R_0 to T_0 quaternary conformational change. This result indicates that the transition state is much more R-like than T-like. It predicts that adding ligands or protons changes the free energy of the transition state by nearly the same amount as the free energy of the R_0 state. This prediction is borne out by the published results on human Hb, which show that changes in the R - T conformational equilibrium are manifested mainly as changes in the T to R rates. The results further show that there is a linear free energy relation between the rates and equilibria, i.e.

$$k(R_i \rightarrow T_i) = \gamma(L_0 c^i)^{\alpha} \quad \text{and} \quad k(T_i \rightarrow R_i) = \gamma(L_0 c^i)^{\alpha - 1}$$

These relations produce the useful practical result that all ten quaternary rates (i = 0 - 4) can be estimated from just 4 parameters: the allosteric equilibrium constant (L_0), the ratio of the ligand affinities of the T and R states (c), the scale factor (γ), and the slope of the linear log-log plot (α) which is 0.2. This value of α suggests that in the quaternary conformational change from R to T the molecule has reached the transition state after proceeding about 20% of the way along the reaction coordinate. A previous theoretical study by Janin and Wodak (Biopolymers 24, 509 (1985)) suggests that a transition state with the subunits packed in a configuration close to the R quaternary structure results from a reaction pathway that maximizes the buried surface area between $\alpha\beta$ dimers.

Tu-Pos215

BETA SUBUNITS OF HEMOGLOBIN IN THE R QUATERNARY STRUCTURE SHOW INDEPENDENT GEMINATE REBINDING AND CONFORMATIONAL RELAXATION KINETICS. Colleen M. Jones, Anjum Ansari, James Hofrichter, Eric R. Henry, and William A. Eaton, Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, MD 20892; Takashi Yonetani, Dept. of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 (Intr. by Rafael D. Camerini-Otero).

The geminate rebinding and conformational relaxation kinetics of singly-liganded and unliganded Hb in the R quaternary structure were compared in partial photolysis experiments on the carbon monoxide complex of an iron-cobalt hybrid Hb, $\alpha(Co)_2\beta(Fe\text{-}CO)_2$, at 20°C. The cobalt porphyrins in the α subunits do not bind carbon monoxide. Partial photolysis creates an anisotropic distribution of molecules, because the laser pulses used for these transient spectral measurements are linearly polarized (see abstract by Ansari *et al.*). The photoselected population rotationally diffuses on the same time scale (τ_R = 30 ns) as the geminate rebinding process (τ = 65 ns), adding a relaxation to the geminate kinetics. This relaxation was eliminated by isotropically averaging the results of separate experiments in which the probe pulse was polarized either parallel or perpendicular to the excitation pulse. In the isotropic data both the geminate yield and relaxation time are independent of the degree of photolysis, indicating that unliganded and singly-liganded molecules have identical geminate rebinding kinetics. Furthermore, spectral changes of the deoxy photoproduct appear to be independent of the degree of photolysis until the R $\rightarrow$ T quaternary conformational change at about 10 μs, suggesting that tertiary conformational relaxation kinetics in unliganded and singly-liganded molecules are also the same. These results are in accord with the MWC allosteric model which asserts that the reactivity of the hemes depends only on the protein quaternary structure and is independent of the number of ligands bound to adjacent heme sites.

Tu-Pos216

THE ROLE OF PHOTOSELECTION IN POLARIZED PHOTOLYSIS EXPERIMENTS. Anjum Ansari, Colleen Jones, Eric R. Henry, James Hofrichter, William A. Eaton, and Attila Szabo. Laboratory of Chemical Physics, NIDDK, NIH, Bethesda, Maryland 20892.

Photodissociation experiments on heme proteins are almost always performed using polarized laser beams, which introduce photoselection effects. Rotational diffusion of the photoselected population changes the apparent ligand saturation, producing an additional process in the kinetics of ligand rebinding. We have developed the theory of photoselection as a function of the excitation laser intensity for an arbitrary absorber and finite pulse-widths. The theory shows that the relative amplitude of the rotational diffusion process decreases with increasing degree of photolysis, but remains significant even at photolysis levels above 90%. To illustrate these effects we performed time-resolved transient absorption experiments on myoglobin (Mb) in 50% glycerol-water with the (10 ns) probe beam polarized both parallel and perpendicular to the (10 ns) excitation beam. The initial anisotropy, $(\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + 2\Delta A_{\perp})$, was obtained at 5°C as a function of the excitation intensity. The measured intensity dependence agrees with the theory, with experimental values at all levels of photolysis which are about 0.9 times the values predicted for a perfect planar absorber. Possible reasons for this slightly lower anisotropy include rapid sub-10ns fluctuations in the orientation of the heme, a tilt in the heme plane following photodissociation, and deviations from perfect planar absorption. In a separate series of experiments, isotropic kinetic curves, i.e. $(\Delta A_{\parallel} + 2\Delta A_{\perp})/3$ versus time, were obtained at the highest level of photolysis from -5°C to 35°C. Small spectral changes in the Soret region of the deoxy photoproduct are observed in the time regime 10 ns - 500 ns. To explain these results, models which include both conformational relaxation and differential rebinding to conformational substates are being explored.

Tu-Pos217

SPECTRAL CHANGES ACCOMPANYING CO RECOMBINATION TO SITE SPECIFIC MUTANTS OF HUMAN MYOGLOBIN. David G. Lambright, Sriram Balasubramanian, Steven G. Boxer. Department of Chemistry, Stanford University, Stanford, CA 94305.

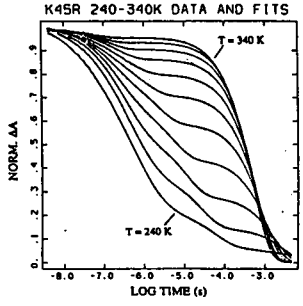
We have investigated the geminate recombination of CO to site specific mutants of human myoglobin at Val68 (Ala, Asn, Ile, Leu), His64 (Ala, Gln), Lys45 (Ala, Arg, Gln), and Asp 60 (Ala, Glu). Transient absorption spectra are monitored on the nanosecond to millisecond timescale following photodissociation of the bound CO with 8 ns laser pulses. The transient spectra are analyzed globally using an approach based on singular value decomposition. As a result of the high signal to noise in these experiments, geminate processes with yields as small as 1% can be observed and analyzed. At room temperature in aqueous solution, a clearly distinguished geminate phase occuring on the nanosecond time scale is observed for all of the mutants except V68I. In addition, we are able to resolve small spectral changes on the time scale of the geminate recombination which correspond primarily to a red to blue shift of the deoxy heme soret band. Although much smaller, these spectral changes resemble those observed in the geminate recombination of CO to iron-cobalt hybrid hemoglobins[1] and geminate recombination of n-butyl isocyanide to myoglobin.[2] We are currently measuring transient spectra in 75% glycerol:water as a function of temperature. Preliminary results at 250K resolve two geminate processes having slightly different spectra. The slower process corresponds to the process III that was first observed in Mb by the Frauenfelder group.[3] As the temperature increases, these processes gradually overlap such that at room temperature they are distinguished only by the spectral shift of the deoxy band. Thus, measurement of transient spectra as a function of temperature for a set of site specific mutants leads to a consistent description of the ligand binding dynamics in these proteins.

[1] Hofrichter, J., et. al. (1985) *Biochem.*, 24, 2667-2679.
[2] Sommer, J. H., et. al. (1985), *Biochem.*, 24, 7380-7388.
[3] Austin, R. H., et. al. (1975), *Biochem.*, 24, 5355-5373.

Tu-Pos218

NANOSECOND CO REBINDING TO RECOMBINANT WILD-TYPE AND MUTANT HUMAN MYOGLOBINS IN GLYCEROL:WATER SOLUTIONS. Sriram Balasubramanian, David G.Lambright, Michael C. Marden* and Steven G. Boxer. Department of Chemistry, Stanford University, Stanford, CA 94305 and *INSERM U299, 94275 Le Kremlin Bicetre, France.

Site-directed mutants of human myoglobin (Mb) have been prepared with a view to studying the effects of several distal residues upon the binding reactions of Mb with various ligands. Here, we present the measurements of CO recombination kinetics to human Mb wild-type and the single site-specific mutants Val 68 -> Ala, Asn, Leu and Ile, His 64 -> Ala and Gln, Lys 45 -> Ala, Arg and Gln, and Asp 60 -> Ala and Glu, in 75% glycerol:water solutions. These experiments were performed over a wide range of time from a few nanoseconds to several milliseconds on a flash photolysis apparatus constructed in this laboratory, with 8 ns pulses and extensive signal averaging capabilities. The transient absorbance was monitored at 440 nm, and the temperature was varied from 240 K to 340 K in 10-degree intervals. The geminate recombination shows two distinct kinetic phases in all the proteins: a fast, non-exponential part which fits well to a stretched exponential, and a slower, exponential part. The figure shows the data recorded for the mutant K45R together with the fits to this function. Both these experimental rate constants show a strong, but very different, dependence on the temperature, allowing the extraction of activation parameters from the data. A very wide range of kinetic behaviour is exhibited by the proteins studied, enabling one to assess the nature of involvement of each of these residues in ligand binding to Mb. The data also permit different models of ligand binding to be tested quantitatively, because the high quality of the data makes it possible to resolve differences between the many models currently in the literature.



Tu-Pos219

ELECTROCHROMISM IN CHARGE TRANSFER AND PORPHYRIN-CENTERED ABSORPTION SPECTRA OF WILD-TYPE AND MUTANT MYOGLOBINS. Dennis H. Oh and Steven G. Boxer, Department of Chemistry, Stanford University, Stanford, CA 94305.

When an external electric field is applied to a sample of non-oriented and immobilized molecules, the resulting change in their absorption spectrum (electrochromism) can often yield estimates of the changes in various electrostatic properties associated with the optical transition, including changes in dipole moments ($|\Delta\mu|$) and polarizabilities ($Tr\Delta\alpha$). Electrochromic experiments on the visible and near-infrared absorption spectra of wild-type sperm whale myoglobin in its met-aqua and met-cyano forms have confirmed that many of the transitions in these spectral regions are associated with changes in the permanent electric dipole moment of the heme. The magnitudes of these changes vary with the exogenous ligand and with the particular electronic states involved. In contrast, the $\pi-\pi^*$ transitions, localized predominantly on the porphyrin, have electrochromic effects which are largely characteristic of non-polar changes, consistent with previous work on related porphyrins in polyvinyl chloride films [Davidsson, Chem. Phys, 45, 409-414, (1980)]. The electrochromic data for the charge transfer transitions also indicate that $\Delta\mu$ lies at a considerable angle with respect to the *transition* dipole moment. More interestingly, site-specific mutations of the human protein which replace valine 68 adjacent to the heme with the negatively charged residues, glutamate and aspartate, significantly perturb the charge transfer spectra [Varadarajan *et. al.*, Biochemistry, 28, 3771-3781 (1989)]. Current work focuses on the electrochromic effects in these mutant proteins in a further attempt to characterize and quantitatively evaluate the effects of electrostatic interactions within a protein matrix.

Tu-Pos220

BLUE SHIFT OF THE SORET BAND OF DEOXY MYOGLOBIN FOLLOWING PHOTOLYSIS SERVES AS A DIRECT SPECTROSCOPIC MARKER FOR CONFORMATIONAL RELAXATION

Kelvin Chu, Pal Ormos †, David Ehrenstein, Hans Frauenfelder, Imre Kovacs, Peter J. Steinbach and Robert D. Young

Department of Physics, University of Illinois at Urbana-Champaign, 1110 W. Green St., Urbana, Illinois 61801

† Institute of Biophysics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary H-6701

The Soret band of carbonmonoxymyoglobin immediately after photodissociation (Mb*) is red shifted from that of deoxy myoglobin (Mb). It is therefore possible to follow the protein relaxation Mb* → Mb by measuring the shift of the Soret band following photolysis at temperatures where the relaxation takes place on a time scale of nanoseconds to seconds.

Carbon monoxide rebinding to myoglobin was measured between 140 K and 250 K over eight time decades. During ligand rebinding a blue shift of the deoxy Soret band was observed above 190 K. The shift is a spectroscopic marker for the Mb* → Mb protein relaxation. This direct observation of the relaxation is compared to the relaxation inferred from the slowdown of CO rebinding above about 160 K.

Tu-Pos221

CO Rebinding Kinetics to the A Substates of Sperm Whale Myoglobin

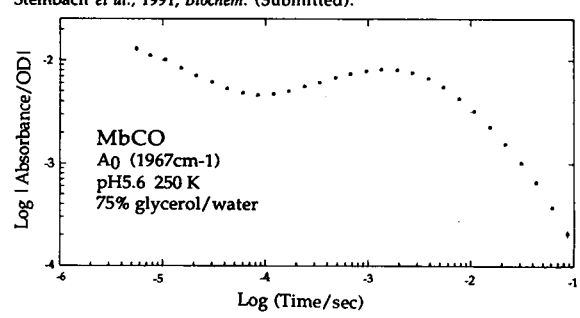
J. Bruce Johnson, Hans Frauenfelder, Don C. Lamb, Robert Philipp, Robert D. Young

Dept. of Physics, U. of Illinois, Urbana, IL 61801

Sperm whale Carbonmonoxymyoglobin exhibits at least three spectroscopically distinguishable carbon monoxide stretch bands between 1910cm^{-1} and 1990cm^{-1}, A_0, A_1 and A_3.[1] The rebinding of these A substates was observed after flash photolysis by monitoring in the infrared at selected wavelengths. The kinetics were measured with a logarithmic timebase digitizer from 5 μs to 1 s and over temperatures ranging from 20K to 300K. The low-temperature kinetics (20K-160K) exhibit increasing rates with increasing temperature which are non-exponential in time due to a distribution of activation enthalpies (g(H)). The three A substates show different g(H) distribuations. At temperatures above 170K ligand rebinding becomes more complicated. From ~170K to ~210K the rebinding rate decreases with increasing temperature.[2] Above 220K, the absorbance change at the peak wavelength of A_0 is non-monotonic (as shown in fiqure) in time suggesting an interconversion of A substates during rebinding.

[1] Ansari *et al.*, 1987, *Biophys. Chem.* 26, 337.

[2] Steinbach *et al.*, 1991, *Biochem.* (Submitted).



Tu-Pos222

KINETIC EXPERIMENTS ON THE PHOTODISSOCIATION OF CARBONMONOXYMYOGLOBIN

Judith R. Mourant, G. Ulrich Nienhaus, Hans Frauenfelder (Intro. by David Braunstein)

Department of Physics, Univ. of Il. Urbana, Il 61801

We have studied the kinetics of the photodissociation of MbCO by monitoring the CO stretch frequencies in the infrared near 1950cm^{-1}. FTIR spectra were taken while slowly photolyzing an MbCO sample at a temperature of 12K, where rebinding is negligible. Depending on the wavelength of the photolyzing light, the dissociation kinetics differ markedly for the three CO stretch bands A_0, A_1, A_3, which correspond to different conformational substates (CS^o) of the MbCO molecules. When photolyzing with wavelengths in the range 620-700nm the rate of photodissociation of substate A_0 is faster than the rate of photodissociation for substate A_1. We will present experimental data and discuss explanations for the observed effect.

Tu-Pos223

Ligand Binding of Heme Proteins: Correlations between Structures, Spectra, and Dynamics.

Aihua Xie, Hans Frauenfelder, Robert Young, and G. Ulrich Nienhaus. Department of Physics, University of Illinois, 1110 West Green Street, Urbana, IL 61801

The protein structure determines both protein function and protein dynamics. We use a conformational marker, band III (~760 nm), to investigate the structural dependence of the ligand rebinding barrier after photodissociation of carbonmonoxy-myoglobin (MbCO) and oxy-myoglobin (MbO_2). Band III is only observed in the deligated state of heme proteins; it is inhomogeneously broadened and shows kinetic holeburning: Myoglobin molecules (Mb*) that have their band III positioned at the red side of the overall band rebind faster than those with their band III at the blue side; the position of band III therefore shifts to higher wavenumbers as ligands rebind. We measured this correlation between the position of band III and the enthalpic barrier for ligand rebinding. We found that such a correlation depends on pH, solvent, ligand (CO or O_2), and heme protein (myoglobin or hemoglobin). We constructed a model which distinguishes between the structural contributions from the distal and proximal parts to the ligand rebinding barriers, and correlates the protein structure, the band III spectra, and the ligand rebinding.

Tu-Pos224

LASER PHOTOLYSIS STUDIES OF PIG AND SPERM WHALE MYOGLOBIN MUTANTS

Theodore E. Carver*, John S. Olson*, Quentin H. Gibson§, Steven J. Smerdon‡, Anthony J. Wilkinson‡
*Dept. Biochem. and Cell Biol., Rice Univ., Houston, TX 77251, §Dept. Biochem., Mol. and Cell Biol., Cornell Univ., Ithaca, NY 14853, ‡Dept. Chem., U. of York, York Y01 5DD U.K.

To date, significant progress has been made in the use of site-specific mutants in the characterization of pathways and intermediates relevant to the binding of ligands to pig and sperm whale myoglobins. Specifically, we hope to describe the nanosecond intermediate "C" and the solvent-to-C barrier in terms of protein structure. Our most recent work has focused on substitutions at positions 68 and 45. Residue 68 lies above the heme near the iron atom, forming part of the hydrophobic interior region of the heme pocket. Substitution of Phe for Val^{68} caused a destabilization of state C, leading to more rapid rates of both bond formation and exit from the protein. This result implies that state C lies in or near the heme pocket and that the bulky benzyl side chain of Phe reduces the number of degrees of freedom available to the ligands occupying this space. We are clarifying this idea with mutants of other residues in the hydrophobic region of the pocket (i.e. Leu^{29}(B10) and Ile^{107}(G8)). Mutation of Val^{68} to Phe also resulted in greatly reduced rates of ligand movement into the heme pocket. This reduction suggests that either 1) preferred pathways for ligand entry lie the back of the heme pocket, or 2) global protein motions are required to permit ligand entry into the pocket.

Residue 45 is close to the distal histidine and outer solvent molecules and has been implicated in a barrier to ligand entry into the heme pocket via the distal histidine. Mutation of Lys^{45} (pig) or Arg^{45} (sperm whale) to polar and acidic residues had little effect upon the movement of gases into the heme pocket while markedly increasing the accessibility of the pocket to bulky isonitriles. Thus, if the distal histidine acts as a gate to regulate access of ligands to the pocket, its interactions with residue 45 do not significantly affect the barrier to the entry of diatomic gases. We are currently examining mutations of Thr^{67}(E10), another external residue postulated to be located along the distal histidine gate pathway. (Supported by the NIH, Robert A. Welch Foundation, a TATP grant from the state of Texas, and the Science and Engineering Research Council, U.K.)

Tu-Pos225

PICOSECOND TRANSIENT RESONANCE RAMAN STUDIES OF IRON PORPHYRINS. Ruth E. Nalliah, Eric W. Findsen, University of Toledo, Toledo, OH 43606.

We present results of studies of the photodynamic behavior of Hemin (Fe PPIX) in two systems, one is Hemin in DMSO (Dimethylsulfoxide) and imidazole, (IM_2 Fe(III)PP) the other is Hemin in DMSO reduced with sodium dithonite ($(DMSO)_2$ Fe(II)PP). Within ~30 ps of excitation, changes in ν_4, ν_1, and the region of ν_2 are observed which suggest the formation of a transient species within 30 ps of excitation in the IM_2 Fe(III) system. These changes appear reversible and are observed using a one pulse protocol with exc. = 416 nm. Intensity changes in other ground state modes have also been observed in this system. The results of studies of the $(DMSO)_2$ Fe(II)PP system indicate that another mechanism, perhaps similar to the power broadening behavior observed in hemoglobin is at work in this system. Reversible spectral changes observed at high photon densities include broadening of ν_4 and broadening and a downward frequency shift of ν_{38} region of the spectrum.

Tu-Pos226

ULTRAFAST MOTION OF HEME-CO IN HEMOGLOBIN

Tianquan Lian, Bruce Locke
and Robin M. Hochstrasser

Department of Chemistry, The University
of Pennsylvania, Philadelphia, PA 19104

The anisotropy of carbon monoxide hemoglobin at ambient temperatures following photolysis of 10% of the sample with 350 fs (FWHM) visible pulses was followed by monitoring the bound CO absorption bleach with an infrared laser from 250 fs to 1 ns. The anisotropy was modeled assuming the porphyrin undergoes small damped harmonic oscillations about in-plane axes along and perpendicular to the plane containing the CO bond and an in-plane dielectric axis of the porphyrin and expressed in terms of the protein rotational diffusion constant, the angle between the CO absorption dipole and the heme plane, the moment of inertia of the porphyrin ring, and the diffusion coefficient of the porphyrin within the protein. The anisotropy is constant at -0.174 ± 0.01 from 250 fs to 1 ns and it appears that the heme plane is rigidly held within the protein as expected from x-ray diffraction studies of crystals. These results indicate that the magnitude of the angle between the CO and the heme plane is $72^{o} \pm 3^{o}$. In addition the geometry determined with picosecond time-resolved infrared spectroscopy is not affected by ultrafast heme motion.

B. L. is a NIH postdoctoral fellow. Support was provided by NIH and the NIH regional laser facility at PENN.

Tu-Pos227

EFFECTS OF SOLVENT VISCOSITY ON
THE MICROSECOND PROTEIN MOTIONS OF MYOGLOBIN
DETERMINED BY PULSED-LASER PHOTOACOUSTICS.

Margaret L. Pearson, Karina Lorenz Mrakovcich, Shane L. Larson and Jeanne Rudzki Small

Department of Biochemistry & Biophysics
Oregon State University, Corvallis, OR 97331-6503.

Pulsed-laser photoacoustics offers a means for measuring the Fe-CO bond strength in carboxymyoglobin, as well as the dynamics of the protein following cleavage of the Fe-CO bond. The technique is sensitive to processes which occur on the nsec and μsec time scales. Preliminary results from our laboratory support a positive value for the enthalpy of formation of the Fe:CO geminate pair, formed within 10 nsec of photolysis of carboxymyoglobin, with little volumetric change in the protein. A subsequent decay occurs, with a lifetime of about 0.8 μsec at 23 °C, resulting in a volume increase and enthalpic change of the protein. This microsecond decay is temperature dependent and appears to be influenced by solvent viscosity. We are interested in this decay as a relaxation process in myoglobin not generally observable through the spectroscopy of the heme. We will present data on the effect of added glycerol or methanol, the source of the myoglobin, the method of deoxygenation, and the intensity of the photolysis laser pulse on the recovered photoacoustic decay parameters.

Supported by NIH grant GM-41415.

Tu-Pos228

TRANSIENT RESONANCE RAMAN INVESTIGATION
OF Ni-mesoHEMOGLOBIN AND Ni-mesoMYOGLOBIN

S.A. Majumder, M.D. Chavez, P.Y.F. Hsu, D.J. Nunez, and M.R. Ondrias
Dept of Chemistry, Univ. of New Mexico, Albuquerque, NM 87131.

Photoproperties of nickel mesoporphyrin reconstituted hemoglobin (Ni-mHb) and myoglobin (Ni-mMb) have been studied by transient resonance Raman spectroscopy. Both Ni-mesoglobins exist as an equilibrium mixture of four- and five-coordinate species, where the major contribution to Ni-mHb and Ni-mMb comes from four- and five-coordinate species respectively. Using selective excitation for specific species within the Soret absorption band, it has been observed that both four- and five-coordinate species of Ni-mHb and Ni-mMb are photolabile. The photodissociative characteristic of the five coordinate species in Ni-mesoglobins is interesting compared to the absence of ligand photolysis (within 10 ns) observed for 5-coordinate Ni-proto globins (nickel protoporphyrin IX reconstituted hemoglobin and myoglobin). The difference in photoproperties between Ni-meso and Ni-proto globins may be related to the difference in the ligand affinity of Ni-mP vs. NiPPIX in various coordinating nitrogenous solvents. (This work was supported by the NIH (GM 33330)).

Tu-Pos229

NEAR-IR ABSORPTION STUDIES OF MODEL HEME COMPOUNDS

M.D. Chavez(a,d), S.H. Courtney(b,c), J.M. Friedman(b,c), and M.R. Ondrias(d)
a) NHLBI, National Institutes of Health, Bethesda, MD 20892 b) Dept. of Chemistry, New York University, New York, NY 10003 c) AT&T Bell Laboratories, Murray Hill, NJ 07974 d) Dept. of Chemistry, University of New Mexico, Albuquerque, NM 87131

Studies on a variety of heme systems provide distinct information on structural parameters that control the reactivity of the heme active site. Band III in the near infrared absorption spectra of heme proteins has been shown to be sensitive to both local structure at the active site and states of reactivity, including ligation and oxidation state. The phenomenon of kinetic hole burning (KHB), relating the inhomogeneous character of band III to functionally distinct species within the system, imply that the distributed structural element(s) responsible for the inhomogeneous line shape of band III is(are) connected with the ligand binding process. Model heme compounds have been used extensively by several groups to isolate the various factors that may control the heme properties. In this study, selected structural elements, i.e. various imidazole compounds, that resemble protein parameters have been incorporated in order to examine the role of the heme in the behavior of band III. For the heme-imidazole-CO complexes, band III has significant dependence on both the proximal ligand as well as the heme geometry. However, no detectable KHB has been detected for any of the model heme complexes, suggesting that the protein is a major factor in the functional consequences of the inhomogeneity of band III.
Supported by NIH GM33330 (MRO).

Tu-Pos230

CONFORMATIONAL DISORDER AND REACTIVITY IN HEMEPROTEINS: A STRUCTURAL MODEL BASED ON RAMAN STUDIES. J.M. Friedman[1], M.R. Ondrias[2], and M. Chance[3]. Departments of Chemistry: (1) New York Univ., (2) Univ. of New Mexico, (3) Georgetown Univ.

The photoproduct absorption band at 760nm (Band III) is inhomogeneously broadened. Kinetic hole burning experiments show that the distribution of barrier heights controlling ligand binding maps onto the inhomogeneous line shape of Band III. This result links functionality directly to the distributed structural parameter responsible for the broadening of Band III. The distributed parameter is likely to be the Fe-heme plane displacement. A complete picture requires knowing what protein structural elements determine this variable distribution of Fe displacements. Both from extensions of KHB and optical pumping techniques to resonance Raman studies of Hb and Mb photoproducts and from RR studies on a very low affinity Hb(1), we have developed a model which links the distribution of Fe displacements to a protein controlled distribution of azimuthal angles for the proximal histidine. (1) Biophysical Chem. (1990) 37, 43-59.

Tu-Pos231

INOSITOL HEXAPHOSPHATE INDUCED pH SENSITIVE CONFORMATIONAL CHANGES OF THE $\alpha 1\beta_2$ INTERFACE OF HEMOGLOBIN. M.J. Lin, M.J. Rao, J.M. Friedman, A.S. Acharya, and R.E. Hirsch. Albert Einstein College of Medicine, Bronx, N.Y. and New York University, Washington Square, New York, N.Y.

β37 Trp (a residue at the $\alpha_1\beta_2$ interface and the primary source of the intrinsic fluorescence of hemoglobin (Hb)) is sensitive to quaternary changes (ie., deoxy→oxy transition), thus serving as a reporter group of structural/conformational changes at this critical region. The sensitivity of this fluorescence signal to structural changes of Hb (ie., DPG/IHP interactions; pH changes; site-specific modifications) has been investigated. Perturbation of the interface by amidation of the γ-carboxyl group of β43 Glu with glycine ethyl ester increases the fluorescence intensity of β37 Trp both in the oxy and deoxy conformation (exc. 280/296nm; em.max. 325nm). This site-specific modification decreases the fluorescence intensity difference of the deoxy→oxy transition compared to the control Hb. The oxygenation-mediated intrinsic fluorescence intensity changes of the unmodified Hb varies with pH with a maximum at pH 6.5. However, when the interaction of IHP with Hb was investigated as a function of pH, the fluorescence intensity exhibited an unusual sensitivity in the acid pH region. In the presence of IHP, below pH 7.0, the fluorescence intensity difference of Hb (deoxy→oxy transformation) decreased compared to the control with the pH sensitive IHP induced fluorescence difference peaking at pH 6.3. The γ-carboxyl of Glu43(β) of oxy HbA is one of the basic carboxyl groups with an apparent pKa of 6.35. When the IHP interaction with the amidated Hb was studied, the fluorescence intensity difference increased considerably, concomitant with deoxy→oxy transition compared to the unmodified protein, suggesting the participation of the γ-carboxyl group of Glu43(β) in modulating the IHP mediated structural change induced at the $\alpha 1\beta_2$ interface. In conclusion: β37 Trp fluorescence reflects (1)site-specific perturbation of the $\alpha 1\beta_2$ interface and (2) the high pH sensitivity of the interface during deoxy-oxy transition (amplified in the presence of IHP) of the modified and unmodified Hbs.

Tu-Pos232

LIGAND-DEPENDENT PERTURBATIONS IN HEMOGLOBIN OBSERVED BY FOURIER TRANSFORM INFRARED SPECTROSCOPY. Sungjo Park and James O. Alben. Department of Physiological Chemistry, Ohio State University, Columbus, OH 43210.

Fourier transform infrared spectroscopy and low temperature photodissociation of carbon monoxide or dioxygen coordinated to hemoglobin have been used to study molecular perturbations at the heme coordination sphere. These have led to assignment of porphyrin E_u and chi vibrational modes in addition to several as yet unassigned bands that may be associated with the proximal histidine. Some of these are overlapped with the putative dioxygen bands that we also observe at 1107 cm^{-1} (cf. Barlow, et al., Bioch. Biophys. Res. Com. 55, 91-95 (1973)) and 1156 cm^{-1}, and show ligand-dependent intensity differences. Under our conditions of continuous light at 10 Kelvin, photolysis was estimated to be 95-98% complete with carboxyhemoglobin, and 33% complete with oxyhemoglobin, in good agreement with room temperature measurements of M. Chance, et al. (Biochemistry 29, 5537-5545 (1990)). The low fractional photolysis of oxyhemoglobin at 10 Kelvin is reasonably described by fast geminate recombination of dioxygen.

This work was supported in part by the National Institutes of Health, grants HL-28144 and RR-01739.

Tu-Pos233

LOW TEMPERATURE FTIR STUDIES OF RECOMBINATION IN OXYMYOGLOBIN *R. A. Lee and M. R. Chance, Department of Chemistry, Georgetown University, Washington, D. C. 20057*

Hemoglobin and myoglobin transport oxygen that is essential for a number of metabolic processes in the body. Although the study of carbon monoxide recombination to heme proteins has been extensive, there is much less known about the intermediates of oxygen binding. In previous studies, we have observed the Soret spectra of MbO_2 and MbCO and their photoproduct yields at 10 K. (Chance, et. al., *Biochem.* 29, 1990, p. 5537.). The photoproduct yield of MbCO on a 30 picosecond to long time scale is 95 ± 5% whereas that of MbO_2 is 40 ± 5%. The fast recombining or (non dissociable MbO_2 population recombines within 30 ps and comprises 60% of the total, while the stable population (no detectable recombination after 10 minutes) comprises 40% of the total. Methods of vibrational spectroscopy (Potter, et al., *Biochem.*, 26, 1987, p. 4699; Bruha and Kincaid, *JACS.*, 110, 1988, p.6006.), clearly indicate multiple bands for MbO_2 ligand stretching modes. However, it is disputed whether these conformations correspond to multiple states of the protein with different reactivity as for CO binding (Chance et. al., *JBC*, 262 , 1987, p.6959.). If the multiple bands have different rebinding kinetics, this supports multiple steric and electronic environments of the distal ligand.

We present results of the low temperature recombination of O_2 with myoglobin using Fourier Transform Infrared Spectroscopy. The bound state of oxymyoglobin exhibits two bands at 1115 cm^{-1} and 1134 cm^{-1} with ca. 2 to 1 intensity ratio. Upon photolysis of this sample only 40% of the sample is stably photolyzed, and the intensity of each peak is reduced equally. After heating to further recombine the sample, the bands reappeared simultaneously. The concurrent recombination of these bands and the 7K photoproduct spectra suggests a single protein conformation as proposed by Bruha and Kincaid. Therefore, it is unlikely that the distal pocket engineers multiple conformations for MbO_2. We will also present results of pH effect on recombination. This research is supported by a grant from the Petroleum Research Fund, #23257-G3.

Tu-Pos234

A COMPARATIVE STRUCTURAL INVESTIGATION OF MYOGLOBIN WITH GASEOUS LIGANDS AND HEAVY ATOM DERIVATIVES WITH EXAFS

K. S. Reddy, K. Zhang and B. Chance
Dept. of Biophys.& Biochem.
University of Pennsylvania Philadelphia PA 19104

The reaction path way of entry and exit of the ligand to the heme pocket and to the Iron atom of myoglobin can be studied at shorter distances by the EXAFS technique and at longer distances by the crystallography and NMR. The present study deals with kinetics and EXAFS which we have been engaged for some years, particularly to the point of establishing the position of the axial ligand immediately after photolysing it. However in order to increase the accuracy with which the position of the axial ligand can be located by EXAFS, an atom of 'Z' value greater than carbon and nitrogen is highly desirable and for that reason we have undertaken the study of ligation of myoglobin with phosphines. Unpublished observations indicate that the phosphines react with ferrous and ferric myoglobins with characteristic optical spectrum. A comparative study of MbO_2 and $MbP(C_2H_5)_3$ indicate that the ' P 'atom gives much better X-ray scattering signal than the 'O'. In addition the preliminary EXAFS data analysis indicate that the Fe - P distance is different in ferrous and ferric Mbs. We consider it advantageous to use such derivatives not only for increased EXAFS visibility but also for X -ray diffraction, high resolution NMR and kinetic measurements. This will offer a convenient way of probing the conformation of the wild and mutant protein and the active site in ferric and ferrous forms.

Tu-Pos235

PHOTOACOUSTIC SPECTROSCOPY STUDY OF THE HYDRATION EFFECTS IN HEMOGLOBIN

Marinônio L.Cornélio, IBILCE-UNESP, S.José do Rio Preto and Rosemary Sanches, IFQSC-USP, S.Carlos, Brazil.

The inter-relationship hydration/structural dynamics/function of a protein has been subjected to a great investigation. In this work the hydration effects in lyophilized human hemoglobin were observed using photoacoustic spectroscopy. Samples of carboxyhemoglobin kept at different relative humidity environments, showed variations in their spectra in the Soret band region. The sample hydration was obtained from the intensity of the water band at 1.95 μm, normalized to the intensity of the band at 2.2 μm that is due to the protein N-H vibrations. This ratio was first calibrated to the water content obtained by weighting and measured for all the samples studied. For the samples which were kept at low hydration (bellow about 0.10 mg H_2O/mg protein) the Soret band appeared at 419 nm, characteristic of the carboxy derivative, whereas at high hydration (above about 0.55 mg H_2O/mg protein) the band was at 414 nm, characteristic of the oxy derivative. In the intermediate region the spectrum was a mixture of both derivatives. This ligand change observed at high hydration may be explained assuming that the protein has flexibility and reaches a conformational state which enables the ligand to go in and out. At low hydrations the protein structure is rigid and such that the access to the heme group is closed and the ligand change impossible. This explanation agrees with other experimental results that indicate the existence of two structures for this protein in solution.