Th-Pos279

CAN MYOSIN HEADS WEAKLY-BOUND TO ACTIN EXERT A DRAG ON FILAMENT VELOCITY? YES AND NO. ((E. Pate, M. Bhimani, G. Wilson, and R. Cooke)) Dept. Math., WSU, Dept. of Biochem. and CVRI, UCSF, and Dept. Anatomy, Univ. Sydney.

We have investigated the effects of vanadate (Vi) on maximum shortening velocity (Vm) in chemically-skinned rabbit psoas fibers as a function of temperature (T). Attached cross-bridges bound to Vi are thought to be an analog of the weakly-bound A•M•D•Pi state. Using new temperature-jump experimental protocols which allow reproducible data to be obtained from fibers activated at high temperature, we have reexamined the effect of increased [Vi] on Vm for T≤30°C. We find that for T≤20°C, increasing [Vi] inhibits Vm; for T≥25°C, increasing [Vi] does not inhibit Vm. Thus our results reconcile the reported differences on the effects of Vi on Vm in the studies of Wilson et al, 1990 (no effect, 25°C) and Chase et al, 1993 (inhibition, 10-13°C). The data suggest that the weakly-bound state can inhibit Vm at low temperature, but not at high temperature. We have also investigated the effects of pH on Vm at 30°C. Previous reports by ourselves and others (T≤15°C) indicate that decreasing pH decreases Vm. At 30°C, however, we find that decreasing pH does not inhibit Vm. Thus pH may be functioning in a manner similar to Vi, populating a weakly-bound state. Additionally, the data at more physiological temperature indicate that previous assertions from studies with glycerinated fibers implicating decreasing pH in the inhibition of Vm during muscle fatigue would appear inapplicable at in vivo temperatures. Supported by NIH grants HL32145 (RC) and AR39643 (EP), a grant from the Muscular Dystrophy Association (RC). EP is an American Heart Association Established Investigator.

SMOOTH MUSCLE PHYSIOLOGY II

Th-Pos280

CHARACTERIZATION OF SMOOTH MUSCLE MYOSIN LIGHT CHAIN (LC) PHOSPHORYLATION, & INTRACELLULAR Ca^{2+} ($[Ca^{2+}]_i$), IN ISOTONIC & ISOMETRIC CONTRACTION. ((He Jiang)) Dept of Physiol, Univ of Manitoba, Winnipeg, MB R3E 0W3 CANADA

We investigated the temporal relationships of $[Ca^{2+}]_i$, LC phosphorylation, isometric force development and isotonic shortening stimulated with electrical (EFS), K^+, and acetylcholine (Ach). Phosphorylation of LC increased rapidly upon EFS (isometric) and peaked at about 4.5 ± 0.7 s. The isotonic LC level showed smaller peak values; a spontaneous elevation in the relaxation phase was seen. The level of $[Ca^{2+}]_i$ (fura-2) and mechanical properties were monitored simultaneously. The intervals between increase in $[Ca^{2+}]_i$ and onset of contraction were 0.8 ± 0.17 for EFS, 2.6 ± 0.54 for K^+, and 1.75 ± 0.3 s for Ach, respectively. The time for the onset of isotonic shortening after increase in $[Ca^{2+}]_i$ was 1.57 ± 0.3 s greater than that for isometric. The levels of $[Ca^{2+}]_i$ peaked by 3.1 ± 0.6 for EFS, 17.46 ± 3.7 for K^+, and 6.49 ± 1.9 s for Ach, respectively. In most cases, force returned to basal level prior to $[Ca^{2+}]_i$, which showed either a sustained submaximal level or a biphasic transient in the late phase of contraction. Isotonic $[Ca^{2+}]_i$ was also less than isometric and a spontaneous increase late in relaxation was noticed in the former. We conclude that LC phosphorylation is very sensitive to small increase in $[Ca^{2+}]_i$ early in both types of contractions. The time between $[Ca^{2+}]_i$ increase and onset of contraction is much faster when Ca^{2+} is released from intracellular source compared to that from extracellular. Furthermore, isotonic shortening is associated with reduced activation of smooth muscle while late in isotonic relaxation spontaneous reactivation develops. (Supported by a grant and a fellowship (H.J.) from Med Res Council, Canada)

Th-Pos281

COMPUTER STOCHASTIC SIMULATION OF SMOOTH MUSCLE CONTRACTION. ((Jizhong Wang* and Newman L. Stephens)) Dept. of Physiol., Univ. of Manitoba, Winnipeg, Canada R3E 0W3

Many investigators have shown that the mechanical properties of various smooth muscles are quite similar to those of striated muscle, which indicates smooth muscle most likely has the same or comparable force generation mechanisms as striated muscle. Little doubt now remains about the applicability of the sliding filament/crossbridge theory to smooth muscle contraction the ambiguity of the cyto-skeletal structure of the smooth muscle cell notwithstanding. In this study, we added a weakly-binding state, under the rationale that weakly-binding crossbridges would act as internal resistances and contribute to muscle stiffness during muscle contraction, to Hai's (1988) four state model to form a more flexible and complete five state, strain-dependent model. To avoid laborious analytical parameter finding, we used Brokaw's (1976) stochastic procedure and modified it to fit our particular needs. Our model allowed us to simulate most if not all of the mechanical properties of an activated smooth muscle. Thus we found: (1) The recovery time of the force transient to quick release was longer than that for quick stretch. (2) Two fast force transients followed by a slow transient on quick release were observed with some variability due to stochastic fluctuations arising from our model simulations. (3) The force-velocity relation obtained by both isovelocity and isotonic shortening methods exhibited the conventional shape. (4) Muscle stiffness preceded force development by 10-30 ms by taking into account the weakly binding state, which offered another possible explanation for the force lag. (5) Finally, myosin light chain phosphorylation increased as did Ca^{+2} concentration in the early phase of contraction; these gradually dropped to a lower level in the later phase, during which force increased and was then maintained at a plateau value. (Supported by the Manitoba Heart and Stroke Foundation, Canada; J.Wang is the recipient of a studentship from the Medical Research Council of Canada).

Th-Pos282

KINETICS OF CONTRACTIONS ELICITED BY PHOTOCHEMICAL INACTIVATION OF THE CALCIUM ENTRY BLOCKER NIFEDIPINE IN DEPOLARISED SMOOTH MUSCLE ((U. Malmqvist and A. Arner)) Dept Physiology and Biophysics, Lund University, Lund Sweden. (Spon. by H. Westerblad)

The kinetics of contractile protein activation and force development was investigated in guinea-pig taena coli smooth muscle using photochemical inactivation of the calcium entry blocker Nifedipine. This compound looses its channel blocking activity after illumination and has previously been used to study the excitation contraction coupling in cardiac muscle by Morad et al. 1983 (Nature 304: 635). Contractions were elicited with 60 or 120 mM K^+ in the presence of Ca^{2+}. Addition of Nifedipine (10^{-9} M) inhibited force to about 20% in the depolarising K^+ solution in the presence of calcium. Illumination with an ultraviolet light flash induced a rapid contraction to almost 100% of the force during the K^+ induced contractions. The force development started after a delay of 300 ms. The delay was independent of extracellular $CaCl_2$. The rate of force development was 0.139 s^{-1} at 60 mM K^+ and 2.5 mM extra cellular $CaCl_2$. At 0.4 mM $CaCl_2$, force was decreased to about 40% and the rate to 0.095 s^{-1}. At 120 mM K^+ and 2.5 mM $CaCl_2$ the rate and force amplitude were similar to those obtained at 0.8 mM $CaCl_2$ at 60 mM K^+. The calcium and K^+ dependence of the rate of contractions reflects modulation of the rate of calcium influx or of the level of intracellular calcium reached after the light flash.

Th-Pos283

MECHANISM OF AGONIST-INDUCED CALPONIN REDISTRIBUTION IN VASCULAR SMOOTH MUSCLE CELLS.
((C.A. Parker+, K. Takahashi**, T. Tao*, and K.G. Morgan+)) +Harvard Medical School, Beth Israel Hospital, Boston, MA 02215; *Boston Biomedical Research Institute, Boston, MA 02214; **Center For Adult Diseases, Osaka, Osaka 537 Japan. (Spon. by D.R. Rigney)

We have reported that agonist stimulation of smooth muscle cells causes redistribution of the thin filament associated protein calponin (CaN). CaN appears homogeneously distributed in resting cells, but in stimulated cells there is a loss of immunoreactive calponin in the core of the cell giving a "donut-like" appearance on cross-section. We have now investigated the mechanism of this redistribution by determining the kinetics and the effect of PKC antagonism. In the presence of 1 μM calphostin, a relatively selective protein kinase C (PKC) inhibitor, the redistribution was inhibited by 44%. The half-time for redistribution of CaN was 3.4 minutes. This is shorter than the half-time for the PKC-associated contraction, 6.4 minutes. The time course of CaN redistribution overlaps with that of PKC translocation. Studies were attempted to identify the structural basis of the donut-like appearance of CaN immunofluorescence in the stimulated cells. CaN distribution was compared to that of actin, myosin and desmin in resting and stimulated cells. Agonist stimulation caused no detectable redistribution of any of these filaments. CaN was distributed similarly to actin and myosin at rest, but differently from that of all three populations of filaments after stimulation. These results suggest a model whereby CaN regulates vascular tone via a PKC-dependent pathway. (Support: NIH HL-42293 and AR-41637)

Th-Pos284

LOADING OF CALDESMON PEPTIDE (GS17C) INTO INTACT VASCULAR SMOOTH MUSCLE BY REVERSIBLE PERMEABLIZATION.

((C.A. Lajoie, C.A. Parker, C.-L. A. Wang, and K.G. Morgan)) Program in Smooth Muscle Research, Harvard Medical School, Beth Israel Hospital, Boston, MA 02215; Boston Biomedical Research Institute, Boston, MA 02214.

GS17C is a peptide that contains the residues from Gly^{661} to Ser^{697} of the caldesmon (CaD) sequence plus an added cysteine at the C terminus. The peptide binds to F actin but does not inhibit actomyosin ATPase activity. It has been shown to cause a contraction of single ferret aorta cells possibly by disinhibiting endogenous CaD (Katsuyama, et al., 1992). To further investigate the mechanism of the contraction caused by this peptide we have extended its use to an intact multicellular preparation. GS17C was first reduced, then labeled with iodoacetamidofluorescein and was loaded into intact ferret aorta strips by the chemical loading procedure previously used for loading aequorin into smooth muscle (Morgan and Morgan, 1982). Successful loading into all cells of the strip was confirmed in all experiments by confocal microscopy, with which the labeled peptide was shown to colocalize with rhodamine phalloidin staining of actin. Incorporation of GS17C into these cells increased basal tone to 167% of control. Basal myosin light chain phosphorylation was found to be 13 ± 2.8% compared to control tissues of 15 ± 0.6%, ruling out an effect of the peptide on myosin light chain phosphorylation. These results are consistent with the idea that GS17C contracts smooth muscle by disinhibition of endogenous CaD and also provide a method whereby this compound can be more broadly utilized. (NIH HL-42293 and AR-41637)

Th-Pos285

DETERMINATION OF $[Ca^{2+}]_i$ REQUIREMENT FOR TRANSLOCATION OF α-PROTEIN KINASE C IN INTACT VASCULAR SMOOTH MUSCLE CELLS.

((R.A. Khalil, C. Lajoie and K.G. Morgan)) Program in Smooth Muscle Research, Harvard Medical School, Boston, Massachusetts 02215.

Because of the inherent difficulties in maintaining physiological conditions in biochemical assays, the quantitative $[Ca^{2+}]_i$ required for activation of PKC in intact cells remains unclear. In the present study, $[Ca^{2+}]_i$ was measured in freshly isolated vascular smooth muscle cells loaded with fura-2 while, in parallel, the distribution of the Ca^{2+}-dependent α-PKC was monitored using digital imaging microscopy. In the presence of 1 mM $[Ca^{2+}]_o$, phenylephrine (PE) caused an initial transient increase in $[Ca^{2+}]_i$ followed by a steady-state increase, and a gradual translocation of α-PKC from the cytosol to the surface over the course of 10 min. Verapamil abolished the PE-induced $[Ca^{2+}]_i$ transient and reduced the steady-state $[Ca^{2+}]_i$ but did not affect α-PKC translocation. The threshold $[Ca^{2+}]_i$ required for α-PKC translocation was determined by changing $[Ca^{2+}]_o$ in steps while monitoring $[Ca^{2+}]_i$. In the absence of PE, increasing $[Ca^{2+}]_i$ caused < 25% of maximal α-PKC translocation. In the presence of PE, maximum translocation of α-PKC occurred at $[Ca^{2+}]_i \geq 198$ nM. The $[Ca^{2+}]_i$-PKC translocation relationship indicated that PE augmented translocation of α-PKC primarily by increasing the slope of that relationship. These results indicate that the $[Ca^{2+}]_i$ threshold of agonist-induced α-PKC translocation *in situ* approaches resting levels and is far less than that reported in most *in vitro* assays; and are consistent with an agonist-induced enhancement of cooperative interactions rather than a change in the affinity of PKC for $[Ca^{2+}]_i$. (Support: NIH HL-31704 and 42293)

Th-Pos286

AN INTRACELLULAR ACIDOSIS IS NOT REQUIRED FOR HISTAMINE OR NH_4^+-INDUCED SMOOTH MUSCLE CONTRACTION

((Xiao-Liang Chen and Christopher M. Rembold)) Cardiovascular Division, (Internal Medicine), University of Virginia, Charlottesville, VA 22908

We examined whether agonist-induced changes in BCECF-estimated intracellular pH (pH_i) alter histamine induced changes in aequorin-estimated intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), myosin phosphorylation (MP), and stress (normalized force) in swine carotid medial tissues. Histamine stimulation (1 μM) induced a rapid increase in $[Ca^{2+}]_i$, MP, and stress associated with a slow development of an intracellular acidosis. Washout of histamine induced a rapid decrease in $[Ca^{2+}]_i$ and stress associated with a slow resolution of the acidosis. Addition of 10 mM NH_4^+ coincident with histamine aborted the acidosis and significantly slowed the histamine-induced increase in $[Ca^{2+}]_i$, MP, and stress. However, this was not observed with three other protocols: 1) addition of 10 mM extracellular $Tris^+$ coincidentally with histamine, 2) increasing pH_o to 7.6 coincidentally with histamine, or 3) addition of 10 mM NH_4^+ ten min prior to histamine all aborted the histamine-induced acidosis, but increases in $[Ca^{2+}]_i$, MP, and stress were not altered. These data suggest that histamine-induced changes in pH_i have little effect on $[Ca^{2+}]_i$-dependent myosin phosphorylation and the resulting contraction in swine carotid. It appears that NH_4^+ alters $[Ca^{2+}]_i$ in smooth muscle by mechanisms in addition to its effects on pH_i. Support: Markey Trust, NIH HL38918, and the Virginia AHA.

Th-Pos287

GENISTEIN INHIBITS $[Ca^{2+}]_i$, STRESS AND MYOSIN LIGHT CHAIN PHOSPHORYLATION IN SWINE CAROTID MEDIA.

((E.M. Gould, C.M. Rembold and R.A. Murphy)) Dept. Mol. Physiol. and Biol. Physics and Dept of Internal Medicine-Cardiology Division, University of Virginia, Charlottesville, VA 22908. (Spon. by John D. Strauss)

Di Salvo and colleagues (Biochem. Biophys. Res. Comm, 190:968-974, 1993) suggested that tyrosine kinase(s) may modulate the Ca^{2+}-sensitivity of contraction in β-escin permeabilized mesenteric microvessels. We examined the effects of the putative tyrosine kinase inhibitor, genistein, on $[Ca^{2+}]_i$, myosin regulatory light chain (MRLC) phosphorylation, and isometric stress in intact swine carotid media tissues to test a potential role for tyrosine kinases in altering the Ca^{2+}-sensitivity in vascular smooth muscle. When stimulated with either 3 μ M or 10 μM histamine, $[Ca^{2+}]_i$ (estimated using the photoprotein, aequorin), MRLC phosphorylation, and stress were decreased ($p < 0.01$) in 30 μM genistein pretreated tissues relative to dimethyl sulfoxide (DMSO) controls. The decrease in $[Ca^{2+}]_i$ quantitatively accounted for the decrease in MRLC phosphorylation and stress. There was no measurable change in the Ca^{2+}-dependence of MRLC phosphorylation with genistein pretreatment. Ca^{2+} mobilization from the intracellular pool would not be a factor in the steady-state measurements examined in this study. The decrease in $[Ca^{2+}]_i$ produced by genistein may be due to a decrease in Ca^{2+} influx and/or an increase in Ca^{2+} efflux through the plasma membrane. Changes in Ca^{2+}-sensitivity do not appear to occur with genistein treatment suggesting a lack of a role of tyrosine kinase phosphorylation in the regulation of Ca^{2+}-sensitization..

This work was supported by a fellowship from the American Heart Association, Virginia Affiliate, Inc. and NIH grants P01 HL19242 and R01 HL38918.

Th-Pos288

LATCH IN PHASIC SMOOTH MUSCLE: MYOSIN PHOSPHORYLATION, AND STRESS IN ELECTRICALLY STIMULATED RABBIT URINARY BLADDER DETRUSSOR AT 37° C.

((S.-C. Kwon, and R.A. Murphy)) Department of Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, VA 22908

The kinetics of phosphorylation of the myosin regulatory light chain (MRLC) and cellular activation in phasic smooth muscle at 37° C are limited by agonist diffusion. Electrical field stimulation (60 Hz, 10 V)-induced contractions were atropine-sensitive and dependent on external $[Ca^{2+}]$. Significant increases in MRLC phosphorylation were detected after 30 msec stimulation and peak values of 0.51 moles P_i/mole MRLC were attained at 700 msec. MRLC phosphorylation then fell to 0.23 moles P_i/mole MRLC by 30 sec of stimulation. The stress response exhibited a latency of 128 ± 24 msec (n=7), reached a maximum of $1.238 \pm 0.107 \times 10^5$ N/m^2 at 7.5 sec and then fell gradually to $0.899 \pm 0.123 \times 10^5$ N/m^2 at 30 sec. Myosin dephosphorylation and relaxation were measured after 10 sec of electrical stimulation. MRLC was dephosphorylated during relaxation with a half-time of 0.6 sec and stress declined with a half-time of 3.1 sec. These data could be modeled by the 4-state crossbridge hypothesis proposed by Hai and Murphy (Am. J. Physiol. 254, C99-C106, 1988) in which Ca^{2+}-dependent MRLC phosphorylation by myosin light chain kinase is the only postulated regulatory mechanism. These results support the hypothesis that contraction of phasic as well as tonic smooth muscle can be explained in terms of Ca^{2+}-dependent activation of myosin light chain kinase, although the kinetics of crossbridge phosphorylation and cycling are much faster in the phasic urinary bladder than in tonic smooth muscles, such as swine carotid and bovine trachea.

Supported by NIH grants HL 19242.

Th-Pos289

LENGTH INDEPENDENCE OF MYOSIN REGULATORY LIGHT CHAIN (MRLC) PHOSPHORYLATION IN THE SWINE CAROTID MEDIAL RING.

((C.J. Wingard, A.K. Browne and R.A. Murphy)) Department of Molecular Physiology and Biological Physics, Health Sciences Center, University of Virginia, Charlottesville, VA 22908.

There is evidence to suggest that both myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) are associated with the myofilaments. Localization of MLCK and/or MLCP may play an important role in determining the rates of (de)phosphorylation of crossbridges. A test of this hypothesis is to determine whether (de)phosphorylation kinetics or steady-state values of MRLC phosphorylation change when the muscle length is altered. The descending force-length relationship in a carotid medial ring has the same slope as that of striated muscle where the fall in force reflects a decrease in overlap of the thick and thin filaments. It is reasonable to hypothesize that changes in length that produce large changes in active force would affect phosphorylation levels if MLCK and/or MLCP are bound to the myofilaments. Ring lengths were adjusted to 0.6, 1.0, 1.4 and 1.8 times the length for maximum force development (L_o). Tissues were then frozen at rest or after 30 minutes of depolarization with 109 mM KCl for determination of steady-state phosphorylation levels (table).

LENGTH (L / L_o)	0.6	1.0	1.4	1.8
Resting (n = 5-14)	5.8 (3.3)	6.4 (0.9)	3.6 (2.5)	2.6 (1.6)
KCl 109 (n = 5-17)	24.9 (7.6)	25.0 (1.7)	24.6 (2.0)	26.7 (4.8)

All values are MRLC phosphorylation in %, (± SEM).

Phosphorylation time course data also exhibited no significant differences: at 30 seconds MRLC phosphorylation was 45.3 (3.0) at L_o and 50.8 (3.8) at 1.8 L_o. The results show that the kinetics of MRLC phosphorylation and activation of the carotid medial ring are independent of length. This work was supported by NIH (5P01 HL19242) and fellowship form the VA affiliate of the American Heart Association.

Th-Pos290

PERMEABILITY OF RECEPTOR COUPLED, PERMEABILIZED SMOOTH MUSCLE TO HIGH MOLECULAR WEIGHT(IgG) PROTEINS

((K. Iizuka, A. P. Somlyo and A. V. Somlyo)), Dept. of Molecular Physiology and Biological Physics, Univ. of Virginia, Box 449, Charlottesville, VA 22908

The introduction of putative contractile regulatory proteins into smooth muscles, that, after permeabilization, retain surface membrane receptors coupled to their effectors is an important approach for determining the mechanisms that can regulate the Ca^{2+}-sensitivity of myosin light chain phoshorylation and contraction. Permeabilization with β-escin allows the transmembrane passage of low molecular weight proteins(calmodulin, 17kDa) and retains coupled receptors(1). The purpose of the present study was to evaluate the extent of transmembrane passage of 150kDa proteins, IgG antibodies and lactic dehydrogenase(LDH), in rabbit portal veins permeabilized with β-escin(50-100μM) for 30min at 23-26°C. The permeabilized strips were incubated in a relaxing solution containing monoclonal mouse anti-smooth muscle α actin antibody(IgG, 1:100) for 120min at 4°C and washed in the relaxing solution for 15min. Subsequently they were treated with secondary antibody, goat anti-mouse F(ab')2, labed with TRITC(1:100) for 120min at 24°C or over night at 4°C and washed again. The cytosol of permeabilized cells showed homogeneous staining, whereas cells in control, α-toxin-treated and intact strips were stained only at the strip edges(damaged cells). The Ca^{2+} sensitizing effect of phenylephrine(100μM) was retained in the permeabilized strips treated with the primary antibody for 120min. During the 30min permeabilization 38.1±3.4%(mean±SE, n=5) and within the next 75min approximately 30% of the total LDH leaked out from the β-escin-treated group but not from the α-toxin-treated group(3.22±1.0%, n=5, p<.01, unpaired 2 tail *t* test). These results indicate that permeabilization with β-escin allows the transmembrane passage of 150 kDa proteins under our experimental conditions that also retain receptor-coupled signal transduction.

(1). S. Kobayashi et al. J. Biol. Chem. 264, 17997-18004, 1989

Th-Pos291

FREE ARACHIDONIC ACID (AA) AND DIACYLGLYCEROL (DAG) ARE INCREASED BY AGONISTS IN SMOOTH MUSCLE ((M.C. Gong, A.V. Somlyo and A.P. Somlyo)) Department of Molecular Physiology and Biological Physics, UVa Medicine School, Charlottesville, VA 22908

The extent of 20 kDa myosin light chain (MLC_{20}) phosphorylation and resultant contractile response of smooth muscle to Ca^{2+} can be enhanced by a G-protein coupled mechanism that inhibits the protein phosphatase that dephosphorylates MLC_{20} (1). The messenger(s) that transmits the message from the surface membrane to the myosin bound phosphatase has not been identified. AA can inhibit the protein phosphatase in situ, and disassociates its subunits in vitro (2). The purpose of the present study was to determine whether agonists that can cause Ca^{2+}-sensitization also release AA, as well as DAG, an activator of PKC. DAG and AA levels were measured by TLC in ^{3}H-AA labeled smooth muscle tissue. Phenylephrine (100 μM, 30 min, at 37°C plus indomethacin and NDGA to inhibit AA metabolism) increased free AA (expressed as % of total ^{3}H-AA incorporated) from 2.7%±0.21 (n=8) to 4.2%±0.56 (n=5, p<0.001), DAG from 1.9%±0.17 (n=11) to 3.1%±0.23 (n=11, p<0.001) in rabbit femoral artery. Using the above protocol, 10 μM U46619 also increased AA from 2.7%±0.43 (n=3) to 4.3%±0.21 (n=5, p<0.01), and DAG from 2.0%±0.08 (n=6) to 3.5%±0.28 (n=7, p<0.01) in rabbit pulmonary artery. The present results show that Ca^{2+}-sensitizing agonists can mobilize both DAG and AA in smooth muscle, but further studies are required for determining whether either or both of these lipids are physiological Ca^{2+}-sensitizing messengers. [Supported by NIH PO1HL19242, Human Frontier Science Program and AHA Fellowship (to MCG).] 1.Kitazawa et al: Proc. Natl. Acad. Sci. USA 88:9307-9310,1991; 2.Gong et al: J.Biol.Chem. 267:21492-21498, 1992

Th-Pos292

BMS-182874 BLOCKS ET-INDUCED CALCIUM MOBILIZATION AND FORCE IN VASCULAR SMOOTH MUSCLE. ((S. Moreland and R. Serafino)) Bristol-Myers Squibb Pharmaceutical Research Institute, Department of Pharmacology, Princeton, NJ 08543.

Endothelin-1 (ET-1) elicits vasoconstriction via activation of the G protein coupled ET_A receptor. The purpose of this study was to determine the ability of the novel, nonpeptide, selective ET_A receptor antagonist, BMS-182874, to block the increase in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) and force resulting from ET-1 stimulation of cultured vascular smooth muscle A10 cells and of circumferential strips of rabbit carotid artery denuded of endothelium. A10 cells and carotids were loaded with the calcium indicator dye, fura-2, by incubation with fura-2 acetoxymethyl ester. Experiments were carried out with a SPEX spectrofluorometer. In resting A10 cells, $[Ca^{2+}]_i = 83 \pm 1.1$ nM. ET-1 caused transient increases in $[Ca^{2+}]_i$ ($EC_{50} = 2.3 \pm 0.6$ nM) that were competitively blocked by BMS-182874 ($K_B = 140 \pm 20$ nM). In the carotid strips, ET-1 prompted an immediate increase in $[Ca^{2+}]_i$ followed by a slower increase in force. Low concentrations of ET-1 produced a monotonic increase in $[Ca^{2+}]_i$ whereas higher concentrations of ET-1 elicited a transient spike in $[Ca^{2+}]_i$ which subsequently fell to maintained suprabasal levels. Non-cumulative concentration response curves to ET-1 were obtained in the presence and absence of 10 μM BMS-182874. BMS-182874 blocked the increases in $[Ca^{2+}]_i$ and force in a competitive manner and with similar potency. Thus, BMS-182874 was a potent competitive antagonist of ET_A receptors in vascular smooth muscle cells in culture and in carotid arteries.

Th-Pos293

SMOOTH MUSCLE CONTRACTION: EFFECTS OF EARLY INTERVENTIONS ON SUBSEQUENT MECHANICAL FUNCTION. ((R.A.Meiss)) Indiana University School of Medicine, Indianapolis, IN 46202.

Mechanical events early in the course of a smooth muscle contraction can have effects that persist into later parts of that contraction (Gunst,S.J. et al., Am J Physiol 205: C467, 1993; Meiss,R.A., J Musc Res Cell Motil 14: 205, 1993). In a series of experiments on isolated rabbit ovarian ligament muscle, electrically-stimulated contractions were begun isometrically; late in the rise of isometric tension, the muscle was released to a light (first) afterload and allowed to shorten isotonically. At a constant time during this shortening, an increased (second) afterload, just sufficient to arrest the shortening, was applied for times ranging from 0.6 to 3.0 sec. Following this variable episode, parameters of contraction (force-velocity curve, rate of isometric tension redevelopment) were measured; those following the shorter application of the second afterload were depressed compared to those after a longer arrest. In a second series, the first afterload was varied from very light (with high shortening velocity) to approximately full isometric (at low velocity), and the remainder of the protocol was followed. Post-arrest contraction parameters showed greater depression with lower first afterloads, but at these forces some of the depression could be reversed by a longer period of arrest. At higher first afterloads, post-arrest parameters did not depend on second-afterload duration. These results indicate that lightly-loaded shortening early in a contraction had a depressant effect later on; briefly preventing the muscle from further shortening partially reversed this effect.

Th-Pos294

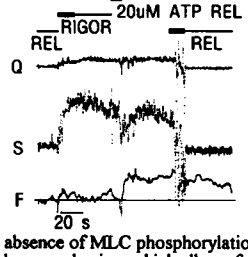
ESTROGEN-INDUCED INHIBITION OF SMOOTH MUSCLE CONTRACTION. ((T. Kitazawa, T Nakajima†, H. Hamada† and Y. Kurachi*)) Dept. of Physiol. and Biophys., Georgetown Univ., Washington, D.C. 20007, † Dept. of Internal Med., Univ. of Tokyo, Japan 113 and *Mayo Clinic, Rochester, MN 55905.

A mechanism of 17 β-estradiol (β-E)-induced inhibition of contraction was studied in various types of smooth muscle. β-E (0.1 - 10μM) reversibly inhibited the contractions induced by high K^+ and phenylephrine, and also inhibited myosin light chain phosphorylation increased by high K^+. There was no significant difference in the inhibitory effects between tissues, animals (rat vs. rabbit) and sexes. This estrogen effect was stereo-specific: diethylstilbestrol > ethinyl estradiol > β-E >> estrone = progesterone = 17 α-estradiol > testosterone. In the absence of $[Ca^{2+}]_o$, phenylephrine-induced transient contraction was not affected by β-E. Pretreatment of ryanodine or thapsigargin to abolish Ca^{2+}-release from the SR had no effect on estrogen-induced inhibition of high-K^+-induced contraction. β-E had neither effect on Ca^{2+}-activated contraction or GTP-induced contractile Ca^{2+}-sensitization in α-toxin permeabilized smooth muscle. β-E did inhibit voltage-dependent Ca^{2+} inward current in isolated cells. These results suggest that estrogen in vitro modulates Ca^{2+} influx through inhibition of L-type of Ca^{2+} channels. This work was supported by NIH RO1 HL51824.

Th-Pos295

WHAT DOES MYOSIN LIGHT CHAIN PHOSPHORYLATION DO IN SMOOTH MUSCLE? ((F.V. Brozovich and M. Yamakawa)) *Bockus Research Institute, The Graduate Hospital,* Philadelphia, PA 19146.

To investigate the mechanism for Ca^{2+} independent force development in smooth muscle, we recorded force and stiffness of α-toxin permeabilized single smooth muscle cells with low concetrations of ATP, ADP and CTP. Rigor (pCa 9) was characterized by high in-phase stiffness (S), but neither force (F) nor quadrature (Q) stiffness, suggesting that the attachment of rigor cross-bridges does not depend on either Ca^{2+} or myosin light chain (MLC) phosphorylation. At pCa 4, 20 μM ATP increased F, S and Q, while 20 μM CTP did not increase F, S or Q, suggesting that although MLC phosphorylation is not required for cross-bridge attachment in a rigor state, MLC phosphorylation is required for cross-bridges to enter force producing states. From rigor, 20 μM ATP (pCa 9) increased F without changing either S or Q. These data suggest that in rigor solution both AM and AM·ADP cross-bridges exist, and ATP induced detachment of AM cross-bridges could be accompanied by AM·ADP cross-bridges undergoing force producing isomerizations. The results of this study imply that in the absence of MLC phosphorylation, smooth muscle contraction could be regulated by a mechanism which allows for an existing population of attached cross-bridges to undergo a force producing conformational change.



Th-Pos296

OKADAIC ACID INCREASES FORCE AND ATPase ACTIVITY BUT NOT MYOSIN LIGHT CHAIN (MLC) PHOSPHORYLATION IN THE ABSENCE OF CALCIUM IN DETERGENT SKINNED SWINE CAROTID ARTERIES.

((Y. Zhang and R.S. Moreland)) Bockus Research Institute, Graduate Hospital, Philadelphia, PA 19146.

The precise role of MLC phosphorylation in the regulation of smooth muscle contraction is unknown. Therefore, we used the protein phosphatase inhibitor, okadaic acid, to determine the effects of MLC phosphatase inhibition on force and actin-activated myosin (AM) ATPase activity in Triton X-100 detergent skinned swine carotid arteries; these measurements were correlated with levels of MLC phosphorylation. The addition of 10 μM okadaic acid during a steady state contraction in response to 7 μM Ca^{2+} had no effect on either force or AM ATPase activity suggesting that MLC kinase/MLC phosphatase activity does not contribute significantly to contractile ATPase activity. In contrast, the addition of 10 μM okadaic acid to an unstimulated artery in the absence of Ca^{2+} induced a slow increase in force to near maximal levels supported by disproportionately low levels of AM ATPase activity. MLC phosphorylation levels were not significantly increased above basal even after 40 min of okadaic acid stimulation in the absence of Ca^{2+}. Steady state force in response to okadaic acid was not affected by the addition of Ca^{2+}, however both AM ATPase activity and MLC phosphorylation levels increased to maximal levels. These results suggest that the contraction in response to okadaic acid is not dependent on Ca^{2+}/calmodulin dependent MLC phosphorylation and are consistent with the hypothesis that okadaic acid allows the expression, presumably by inhibition of a protein phosphatase, of an inherent basal level of smooth muscle AM ATPase activity and a resultant slow increase in force Supported, in part, by NIH HL 37956 (RSM) and a fellowship from AHA SEPA affiliate (YZ).

Th-Pos297

VANADATE OXIDATION ELICITS A CONTRACTURE IN SKINNED GUINEA PIG TAENIA COLI INDEPENDENT OF MYOSIN LIGHT CHAIN (LC_{20}) PHOSPHORYLATION.

((J. Lalli, K. Obara and R. J. Paul)) Department of Physiology & Biophysics, University of Cincinnati College of Medicine, OH 45267-0576 (Sponsored by J. A. McCray)

We previously showed (Biophys.J. 64: A257, 1993) that pre-incubation in high concentrations (mM) of VO_4 elicits a contraction without Ca^{2+} upon washout of VO_4. We further characterized this contraction by measuring LC_{20} phosphorylation, velocity, reversibility and response after thiophosphorylation. Control forces in 6.6 μM Ca^{2+} were 0.5 - 2 mN. Fibers pre-incubated in 4 mM [VO_4], then transferred to a Ca^{2+}-free solution developed ~70% of the control force but showed little LC_{20} phosphorylation (< 7% ≈ control). Subsequent transfer to 6.6 μM [Ca^{2+}] increased force but the total never exceeded the initial control force in Ca^{2+}. Moreover, after maximal phosphorylation of LC_{20} with ATPγS, treatment with VO_4 did not increase force. Fibers had a control velocity of shortening (V_{max}) of 0.15 L_o/sec in Ca^{2+}. After pre-incubation in VO_4, V_{max} was 0.98× and increased to 2.2× control with addition of 6.6 μM Ca^{2+}. After ATPγS, V_{max} was 1.9× control and neither VO_4 pre-incubation nor the addition of Ca^{2+} after VO_4 had any effects on V_{max}. Fibers pre-incubated in 4 mM [VO_4] and 25 mM [DTT] did not produce force. VO_4-contracted fibers could be returned to the initial control state with sustained exposure to DTT, indicating no irreversible damage. The mechanism of activation by VO_4 oxidation is unknown, but may be either a direct effect on smooth muscle myosin or modifications of the regulatory proteins. However, the lack of VO_4 effects on either force or V_{max} in thiophosphorylated fibers would imply a mechanism parallel to that of LC_{20} phosphorylation. Our results suggest a mechanism whereby oxidation injury could increase smooth muscle sensitivity. Supported by NIH HL23240, HL22619 and TG HL07571.

Th-Pos298

EFFECTS OF HYPOXIA ON $[Ca^{2+}]_i$, pH_i AND MYOSIN LIGHT CHAIN (LC_{20}) PHOSPHORYLATION IN GUINEA PIG TAENIA COLI.

((Richard J. Paul, Peggy Sue Bowman, Yukisato Ishida and Kazuo Obara)) Dept. of Physiology and Biophysics, University of Cincinnati College of Medicine 45267-0576.

Hypoxia (N_2 bubbling) reduces the force of a KCl (40 mM) contracture to <10% of control. We previously (J. Physiol. 424: 41, 1991) proposed that this was due to an overall energy limitation, as PCr content and ATP synthesis were also reduced to ~10% and 30% of control, respectively. However, as is typical in most tissues studied under hypoxia, ATP remained at ~50% of control. We further tested our hypothesis using ratiometric fluorescent dyes fura-2 and BCECF to measure $[Ca^{2+}]_i$ and pH_i, and LC_{20} phosphorylation with IEF-PAGE and immuno-western identification. $[Ca^{2+}]_i$ increased with KCl (100% = peak; Ca^{2+}-free baseline = 0%), and steady state after 15 min was 88.9%. Hypoxia then increased $[Ca^{2+}]_i$ to 100.9% with a steady state of 86.4%; reoxygenation decreased $[Ca^{2+}]_i$ to 80.0%. Basal pH_i was 7.23 and increased to 7.36 with KCl. After hypoxia pH_i was unchanged, despite an increase in both lactate content and production. LC_{20} phosphorylation increased from 8.4% in Ca^{2+}-free solution to a peak value of 35.7% in KCl. The steady state value in hypoxia of 27.2% ± 0.8% was not different from that in normoxia (28.8% ± 1.2%). Thus under hypoxia, the major activation pathway of Ca^{2+} and MLC-P_i is not affected, and changes in pH_i are minor. P_i does increase (from 2 to 5 mM), but would appear below that required for significant relaxation in permeabilized fibers. Thus hypoxic relaxation in the taenia coli appears to be directly related to energy limitation, rather than any oxygen sensing mechanism, supporting our previous hypothesis. Supported by NIH 23240, and AHA 92007130.

Th-Pos299

INFLUENCE OF CHANGES IN OSMOTIC PRESSURE ON PRESSURE-INDUCED MYOGENIC TONE IN A RAT CEREBRAL ARTERY.

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It has recently been shown that dihydropyridine (DHP) sensitive calcium channel currents in rat basilar artery myocytes are stretch sensitive (Langton, (1993) *J. Physiol.*, 471: 1-11). Hypo- and hyper-osmotically induced changes in cell volume increased and decreased, respectively, the current amplitude. In view of the sensitivity of pressure (stretch)-induced myogenic tone to DHP-channel antagonists, we have examined the effects of osmotic manipulation on pressure-induced myogenic tone in rat middle cerebral artery. Arteries were pressurized to 70 mmHg in Halpern-pressure myograph. Vessels subsequently developed myogenic tone, their diameter spontaneously decreasing by 57±12 μm from an initial diameter of 232±6 μm. Arteries were superfused with a standard bicarbonate buffer (osmotic pressure 279±3 mosmol l^{-1}) which was then replaced by either a hyposmotic (less 30mM NaCl; 216±4 mosmol l^{-1}), a hyperosmotic (plus 60mM sucrose; 318±3 mosmol l^{-1}) or an isosmotic (less 30mM NaCl, plus 60mM sucrose; 283±6 mosmol l^{-1}) solution and artery diameter monitored.

solution change	change in diameter (μm)	(n)
normal to isosmotic to normal	-11±2 / +12±3	4
normal to hyperosmotic to normal	+25±8 / -17±2	5
normal to hyposmotic to normal	-26±4 / +38±5	4

Hyperosmotic superfusion caused reversible vasodilation, while hyposmotic superfusion (NaCl reduction), constricted. There was also a small constriction to NaCl reduction, under conditions where osmotic pressure was maintained at control values by addition of sucrose. These results demonstrate that altering cell volume (and hence cell stretch), by osmotic manipulation, produces alterations in vascular diameter consistent with previously described osmotic effects on calcium currents. This phenomenon may participate in the control of pressure-induced myogenic tone. *Supported by the British Heart Foundation.*

Th-Pos300

ELECTRICAL GEOMETRY OF UTERINE MYOCYTES.

((M.B. Boyle)) Dept. Ob/Gyn, The University of Texas Medical Branch, Galveston, TX 77555-1062.

The onset of labor is heralded by an impressive repertoire of changes related to enhancement of electrical and contractile mechanisms in uterine muscle cells. Voltage-dependent inward currents increase, different potassium currents are up- and down-regulated, and electrical coupling between muscle cells through gap junctions is greatly increased. We here report that the electrical geometry of the uterine cells may become more complex during late pregnancy. Two groups of normal, untreated pregnant rats were studied: days 19-20 ("pre-term", n=12 cells, 5 animals) and days 22-23 ("term", n=22 cells, 6 animals) As a measure of the electrical geometry of the cells, we analyzed the capacitive transient during a 10-mV voltage-clamp step. The average cell capacitance was not significantly different for the two groups (76 v. 85 pf). In assessing our whole-cell recordings from longitudinal myocytes, we found that in only relatively few cases was the capacitive transient fit adequately by a single exponential, and that these cells tended to be from the day 19-20 (5 of the 12 cells) rather than the day 22-23 (6 of 22) group. The degree of "complexity" may be partly size-related; the day 19-20 cells which were successfully fit by a single exponential were the smaller cells (all < 80 pf). For day 19-20, one-exponential time constants ranged from about 0.1 ms to 0.6 ms; one-exponential time constants for day 22-23 cells exceeded 0.8 ms. The simplest interpretation of this data is that the day 22-23 have more significant internal resistance, due possibly to overall increased length or decreased width, or possibly locally increased access resistance as is associated with T-tubules in skeletal muscle. These data also suggest that, at term, excitation may penetrate into some parts of the cell slowly and less completely than others. A rigorous assessment will require more information about the limiting factors for propagation, both temporal and spatial, within and between cells. However. these findings may have important implications for spread of electrical excitation.

Th-Pos301

AUTOREGULATION OF ARTERIAL CONTRACTIONS: MEMORY IN VASCULAR SMOOTH MUSCLE.

((P.H.Ratz and F.A. Lattanzio, Jr.)) Department of Pharmacology, Eastern Virginia Medical School, Norfolk, VA 23501.

Receptor stimulation of vascular smooth muscle not only causes contractions, but also may initiate a process whereby subsequent stimulations produce weaker contractions (tachyphylaxis). Receptor down-regulation is partly responsible for tachyphylaxis. However, whether tachyphylaxis reflects additional autoregulation of cell activation that lasts only a short time remains to be determined. To examine this possibility, strips of rabbit femoral arteries were contracted for 30 min with sub- (0.1μM) and supra-maximum (10 μM) concentrations of the α-adrenoceptor agonist, phenylephrine (PhE), then relaxed by washing in the presence of an α-adrenoceptor blocker for 10 min. After these pre-exposures, tissues were contracted with KCl to bypass receptors, and active stress (S/So), the extent of myosin light chain phosphorylation (MLCp/MLC) and $[Ca^{2+}]_i$ (F_{340}/F_{380}; fura-2/AM) were measured at 5 min (Table). Negative modulation produced by 30 min pre-exposure to 10 μM PhE was reversible (after washout durations of 30, 60 and 120 min, KCl-induced contractions were, respectively, 0.41, 0.62 and 0.75 S/So). These data suggest that femoral arterial muscle retained short-term (~2 h) memory of receptor activation. Furthermore, memory may have reflected autoregulatory mechanisms invoked by receptor activation involving both positive and negative modulation of post-receptor signal transduction and contractile protein activation.

	S/So	MLCp/MLC	F_{340}/F_{380}
Control:	0.94 ± 0.03	0.38 ± 0.02	0.53 ± 0.09
After 10 μM PhE:	0.39 ± 0.04	0.26 ± 0.02	0.27 ± 0.07
After 0.1 μM PhE:	1.05	0.44	-

Th-Pos302

EFFECTS OF EXTRACELLULAR CALCIUM ON ENERGETIC AND MECHANICAL PROPERTIES OF RAT ANOCOCCYGEUS ((J. S. Walker, I. R. Wendt, and C. L. Gibbs)) Department of Physiology, Monash University, Wellington Rd, Clayton, VIC 3168, Australia. (Sponsored by J.D. Strauss)

Changes in smooth muscle intracellular calcium play a central role in the regulation of smooth muscle contraction and are thought to be mediated in part by extracellular calcium influx. We measured the isometric stress, the isometric heat rate, and the unloaded shortening velocity (V_{wo}) of the rat anococcygeus muscle when the extracellular calcium concentration ($[Ca^{2+}]_o$) was 0.5, 1.25, 2.5 or 5 mM. All experiments took place at 27°C and contractions were elicited by electrical stimulation at 10 Hz.

An early peak in heat rate was depressed in the lower calcium concentrations from 6.2 ± 1.3 mW/g in 5.0mM Ca^{2+} to 4.2 ± 0.7 mW/g in 0.5 mM Ca^{2+}. Significant decreases in the early heat rate were accompanied by a reduction in the rate of rise of force. The steady state stress increased with increasing $[Ca^{2+}]_o$ up to 2.5 mM Ca^{2+}. Economy, the ratio of stress to heat rate, showed no systematic change. An early peak in V_{wo}, 0.48 ± 0.04 L_o/s at 10s in 5.0 mM Ca^{2+}, was both reduced and delayed by lowering $[Ca^{2+}]_o$, 0.40 ± 0.04 L_o/s at 60s in 0.5 mM Ca^{2+}. The steady state V_{wo} in 0.5 mM Ca^{2+} was decreased compared to other $[Ca^{2+}]_o$.

These data suggest that reducing $[Ca^{2+}]_o$ interferes with an early rapid mobilisation of crossbridges but that it has little effect on steady state properties.

Th-Pos303

ROLE OF CYTOSKELETAL PROTEIN PHOSPHORYLATION IN THE REGULATION OF CANINE TRACHEAL SMOOTH MUSCLE CONTRACTION. ((F.M. Pavalko, L. Adam, M.F. Wu, T.L. Walker and S.J. Gunst)) Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, IN 46202.

Contraction of smooth muscle tissues requires that the force generated by actin and myosin be applied the plasma membrane of individual smooth muscle cells. Several cytoskeletal proteins which are localized at smooth muscle dense plaques may participate in the attachment of actin filaments to the membrane. To better understand how actin-membrane attachments are regulated we have examined the phosphorylation state of several actin-membrane linker proteins including talin, vinculin and paxillin during contraction of ^{32}P-labeled canine tracheal smooth muscle strips. Immunoprecipitation of these proteins from muscle extracts stimulated with Ach (10^{-3}M) for various times has provided the following information: 1) talin is phosphorylated at a low basal level in resting muscle and undergoes a rapid increase in phosphorylation reaching maximal levels 1-3 min following stimulation; 2) phosphorylation of vinculin is not detectable in either resting or stimulated muscle; and 3) paxillin is very heavily phosphorylated in resting tissue but does not appear to undergo any change in phosphorylation during contraction. Our results support a role for talin phosphorylation in regulating the attachment of actin filaments to the membrane at dense plaques. In contrast, changes in phosphorylation do not appear to play a role in the regulation of vinculin or paxillin function during Ach-stimulated contraction. Phosphorylation of talin may affect its ability to interact with actin filaments, with vinculin or with the cytoplasmic domain of integrins in dense plaques. Support by the American Lung Association (FMP) and American Heart Association, Indiana Affiliate (FMP) and NIH HL29289 (SJG).

Th-Pos304

THE DIFFERENTIAL EFFECT OF MgADP ON RELAXATION FROM ISOMETRIC CONTRACTION INDUCED BY PHOTOLYSIS OF DIAZO 2 IN TONIC AND PHASIC SMOOTH MUSCLE ((A.S.Khromov,A.V.Somlyo and A.P.Somlyo)) Department of Molecular Physiology and Biological physics,UVA, Charlottesville, Va22908

The rate of detachment of rigor cross-bridges by ATP is more sensitive to slowing by MgADP in tonic (rabbit femoral artery;RFA) than in phasic (rabbit bladder;RBL) smooth muscle[1].The purpose of the present study was to determine the effect of MgADP on the kinetics of relaxation from Ca-activated contraction.a-toxin permeabilized preparations of RFA and RBL were isometrically contracted in the presence of 1.0-1.5 mM Diazo2 and total [Ca] sufficient for producing 40-50% of maximum tension.Relaxation was initiated using 50 ns pulses of near UV light to photolyse the Ca chelator,Diazo2.After photolytic release of the Ca chelator (K_D = 2.2 μM before and 73 nM after photolysis[2])the preparations relaxed almost completely (> 87%).The half-times of relaxation in the presence of 20 mM creatine phosphate (CP) (no added MgADP) were 88.4±2s for (RFA) (n=14) and 19.8±1s for (RBL) (n=7).Removal of CP from the activating solution slowed the relaxation of RFA to130±13s (n=9),but had no significant effect on RBL:19.3±2s (n=7).We conclude that,as found during relaxation from rigor,the effect of MgADP is more pronounced in tonic than in phasic smooth muscle. The higher affinity of MgADP for crossbridges of tonic smooth muscle [1,3] may contibute to their slower kinetics [1]A.Fuglsang et al,J.Mus.Res.&Cell.Mot.(1993)in press.[2]S.R.Adams et al,J.Am.Chem.Soc(1989),111,7957.[3].A.P.Somlyo,J.Mus.Res.&Cell.Mot.(1993) in press.

Th-Pos305

ISOTONIC MYOGENIC RESPONSE OF ISOLATED GUINEA-PIG (GP) LARGE PULMONARY ARTERY. *((X. Su, H. Jiang, and J. Belik)) Departments of Pediatrics and Physiology, Univ. of Manitoba, Winnipeg, MB, Canada R3E 0W3.*

We have previously shown that the GP large capacitance pulmonary artery (PA) exibits a powerful myogenic response (MR) under isometric conditions. Because the control of vascular resistance is dependent on vessel diameter changes caused by muscle shortening, we evaluated the stretch-induced shortening in 2nd order PA from 7 adult GPs'. At the optimal muscle length (L_o), electrical stimulation induced a maximum shortening of (ΔL_{max}) of 22 ± 4% of initial length (L_i) and a stress of 12.6 ± 1.9 mN/mm² (Mean ± SE). The vessel segments were then stretched by from L_o to 100 % of L_o for 3 s each, by increasing the load applied to the muscle. This was followed by an instantaneous decrease in the load to a quasi-zero level. The MR was measured as the change in L_i (ΔL_i) following stretch and further expressed as a % of L_i (%ΔL_i) and ΔL_{max} (% ΔL_{max}). **RESULTS:** Stretch by 40% or more of L_o induced a significant MR (Table) and the MR increased with the degree of vessel stretch. In conclusion, we have demostrated for the first time stretch-induced isotonic shortening in large pulmonary artery of GP. The importance of these findings to the control of pulmonary vascular resistance deserves further investigation (supported by a grant from MRC, Canada).

TABLE

	% STRETCH				
	20	40	60	80	100
%ΔL_i	0.26 ± 0.14	0.38 ± 0.12	0.62 ± 0.12*	1.8 ± 0.68**	2.6 ± 0.93**
%ΔL MAX	1.7 ± 1.3	2.5 ± 1.6	3.5 ± 1.7*	8.0 ± 2.8**	10.6 ± 3.0**

*, **: P< 0.05 & 0.01 as compared with non-stretched values.

MOTILITY OF NONMUSCLE CELLS

Th-Pos306

CALCIUM DISTRIBUTIONS IN MOTILE, ELECTROTACTIC FISH KERATOCYTES MEASURED BY TWO-PHOTON EXCITED FLUORESCENCE MICROSCOPY. ((Ingrid Brust-Mascher†, Rebecca M. Williams*, and Watt W. Webb†)) †Applied & Eng. Physics and *Physics, Cornell University, Ithaca, NY 14853.

Internal calcium concentrations were imaged in fish keratocytes, which extend broad, submicron thin, actin-rich leading lamellipodia and move rapidly in culture. When subjected to an electric field they tend to align and migrate toward the cathode. 3-dimensional submicron spatial resolution was obtained by two-photon ultraviolet excitation using a red, mode-locked, 100 femtosecond pulsed laser (Denk et al., 1990). The intracellular calcium activity was determined by loading cells with both the AM and salt versions of indo-1 and using fluorescence ratios to eliminate dye concentration and cell thickness errors. Calcium concentrations were typically 100-500 nM. We observed non-uniform calcium distributions within the lamella, that were correlated to cell motion; turning cells tended to pivot around slower regions in their lamellae which were higher in calcium. When cells were subjected to electric fields of about 10V/cm, calcium concentrations increased up to five fold within 10 to 30 sec and returned to pre-field values in 30 to 60 sec. Subsequently about 50% of the cells exhibited smaller irregularly spaced calcium spikes. Cells turning towards the cathode had the same kind of non-uniform distributions within the lamella as spontaneously turning cells.

Supported by grants from NSF(DIR8800278) and NIH(RR04224) to the Developmental Resource for Biophysical Imaging and Opto-Electronics.

Th-Pos307

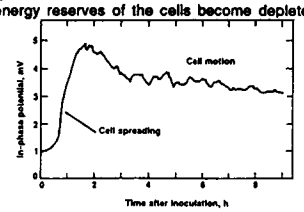
LASER MICROBEAM TECHNIQUE AS A TOOL FOR MICRODISSECTION OF MYOFIBRILLAR BUNDLES AND FUSION OF SARCOLEMMA VESICLES OF SKELETAL MUSCLE. ((C. Veigel, R. Wiegand Steubing and R. Fink)) II. Physiology, INF 326, University of Heidelberg, D-69120 Heidelberg Germany.

The laser microbeam technique was used to selectively prepare fragments of myofibrillar bundles of very small dimensions, and to induce fusion of sarcolemma vesicles. Using a highly focused UV laser microbeam very small (<3μm) myofibrillar fragments with an intact sarcomere striation pattern were obtained. The myofibrillar bundle shortened due to the development of calcium-activated force when small amounts of Ca^{2+} were released in the vicinity of such a fragment by laser-photolysis of the photolabile Ca^{2+}-chelator nitr-7. Very small selected areas from myopathic single muscle cells could also be dissected with a unmatched precision. Furthermore, we prepared sarcolemma vesicles of skeletal muscle fibres to test the effects of the laser microbeam on the muscle membrane directly. Such vesicles could be selectively perforated with single laser pulses to allow entry of the fluorescent marker FITC-dextran. Adjacent vesicles were caused to fuse by a few pulses at low intensity of the laser microbeam. This degree of precision and selectivity is not attainable with other techniques. The laser microbeam thus offers unique advantages in the study of subcellular contractile mechanisms as in membrane research on normal and myopathic muscle.

Th-Pos308

THE RELATIONSHIP BETWEEN MAMMALIAN CELL METABOLISM AND MOTION MEASURED ELECTRICALLY. ((C.R. Keese, C.M. Lo, and I. Giaever)) Rensselaer Polytechnic Institute, Applied Biophysics, Inc., Troy, NY 12180.

We have developed a new method to monitor the morphology and motions of cells in tissue culture. In electric cell-substrate impedance sensing (ECIS), cells are cultured on small (.001 cm sq) gold film electrodes whose impedance is measured with a 1 microamp current at 4000 Hz. When cells spread out on these electrodes they block some of the area, as the current must flow around the cells; this results in large impedance changes. Furthermore, the constant motion of the cells produces fluctuations in the impedance, and changes in cell morphology on the scale of nanometers can be measured (Giaever and Keese, PNAS 88, 7896-790, 1991). When cells are exposed to temperature changes, we have observed an approximate two fold reduction in the power spectrum of the fluctuations for a drop in temperature of 10°C. In other studies the power spectrum of the fluctuations was measured as confluent cell layers were deprived of glucose and all other reduced carbon. The fluctuations remain unchanged until the energy reserves of the cells become depleted which takes about 20 hrs. Following this, there is a gradual decline in the power over the next 25 hours. At this point if glucose is added to the cell medium, the fluctuations rapidly return. These results demonstrate that the ECIS technique can be employed to follow the metabolic activities of cultured cells (Lo, Keese, and Giaever, Exp. Cell Res. 204, 102-109, 1993).



Th-Pos309

ELECTRIC FIELD INDUCED GRADIENTS OF CELL-SUBSTRATUM ADHESION. ((M.J. Brown and L.M. Loew)) Dept. of Physiology, University Of Connecticut Health Center, Farmington, Connecticut, 06030.

DC electric fields induce cathode-directed migration in NIH/3T3 fibroblasts. Using interference-reflection microscopy (IRM) and digital image processing, we have examined the dynamics of cell-substratum adhesion patterns during the galvanotactic response of these cells. Applied electric fields as low as 0.1mV/μm rapidly alter cellular adhesion patterns. Within seconds of field application, a broad wave of increased cell-substratum proximity crosses the cell from anode to cathode. Distinct close and focal contacts are not disrupted, but often become displaced slightly toward the cathode. These initial events precede any gross morphological changes or directional migration. In addition, these effects appear to be proportional to field strength and elastic in nature, as the immediate removal of the field leads to the relaxation of adhesion patterns to their pre-field state. Continuous E-field application results in cathode-directed cellular locomotion. Directionally migrating fibroblasts exhibit a distinct adhesion gradient, with cell-substratum proximity increased toward the cathode and decreased toward the anode. These observations suggest that electric field induced asymmetries of cell substratum proximity may lead to polarized cellular adhesion and directional migration.(supported by USPHS grant no. ES05973)

Th-Pos310

MOTIONS OF TRACTION FORCES, SITES OF CELL-SUBSTRATUM CONTACT, AND ACTO-MYOSIN STRESS FIBERS IN FIBROBLASTS. ((K. Burton, F. Lanni, D.L. Taylor)). Center for Light Microscope Imaging, Carnegie Mellon University, Pittsburgh, PA, 15213.

We have investigated the dynamic spatial relationships of traction forces, focal contacts, and cytoskeletal fibers containing myosin II in living mouse fibroblasts. The locations and relative magnitudes of traction forces were monitored using a modification of the method introduced by Harris (*Science* **208**:177, 1980) in which cells are cultured on transparent elastic silicone. In order to monitor forces applied by relatively weak cells, we have employed polysiloxanes which are significantly more compliant than those reported heretofore. We are able to control the compliance of these silicone sheets appropriate to a wide range of traction forces. Cells adhere well to these substrata which also possess improved optical properties permitting high quality interference reflection microscopy (IRM). Methods of using various types of distortions visualized in the substratum to quantitate applied traction forces are under development.

Structural dynamics of individual cells were studied using three modes of timelapse microscopy: IRM to monitor sites of focal contact at which the cytoskeleton is mechanically coupled to the substratum; Nomarski DIC to obtain high resolution images of cell structure and to visualize substratum distortions produced by traction forces; fluorescence microscopy to reveal acto-myosin fibers into which a rhodamine-myosin analog had incorporated following microinjection of the cells cultured on silicone substrata. Visualization of cytoskeletal fibers was improved by inducing cells to flatten in response to low levels of serum (0.2% v/v). Under these conditions the cells are nearly stationary, but many cytoskeletal fibers are observed to transport roughly perpendicular to their long axes at speeds of 10-20 μm/hr (Giuliano and Taylor, *Cell Mot. & Cyto.* 16:14, 1990). These conditions additionally cause a significant reduction in average traction force, despite an increase in the number of stress fibers. We have studied interactions between stress fibers, focal contacts, and sites of traction forces by monitoring their movements. We find that sites at which traction forces are applied correlate strongly with focal contacts and termini of a subpopulation of myosin-containing fibers, but that these do not exhibit movements corresponding to those of transported fibers. These results suggest that stress fiber transport requires one or both ends of the fibers to be unrestrained by mechanical connections to the substratum, thus allowing lateral movement in response to forces within the cell.

Th-Pos311

NON-MUSCLE MOTILITY: FORCE, VELOCITY AND STIFFNESS IN A RECONSTITUTED FIBROBLAST FIBER. ((Kazuo Obara, Michael Kolodney, Primal de Lanerolle and Richard J. Paul)) Depts. of Physiology & Biophysics, Univs. of Illinois at Chicago, Washington U. & Cincinnati Colleges of Medicine, 45267-0576.

Recent studies (J. Cell Biol. 117: 73, 1992) have shown that cultured cells grown in a collagen matrix reconstitute into a fiber-like structure in which isometric force could be directly measured. We have extended these studies to characterize the contractile properties of NIH 3T3 fibroblasts, including force-velocity relations and stiffness. Calf serum (CS), thrombin and nocodozole all elicited reversible contractures suggesting that both myosin- and microtubule-based motile systems are likely to be involved. Cytocholasin D reduced all forces to near baseline levels, indicating that the mechanical properties were attributable to the 3T3 cells. Calf serum elicited a dose-dependent contraction with a time to peak force of 21.5 ± 1.3 min at 37 °C. CS produced the largest isometric forces of 101.8 ± 15.1 μN, approximately 0.2-1 mN/mm^2. With imposition of constant shortening velocities, force declined with time, yielding time dependent force-velocity relations. Force at 5s was well fit by the hyperbolic Hill equation, V_{max} was 0.037 ± 0.008 L_0/s and the curvature parameter, a/F_0 = 1.09 ± 0.11. V_{max} is similar to those for tonic smooth muscles, but a/F_0 is higher than most muscles. Stiffness, measured by imposition of rapid (<1 ms) shortening steps, averaged 0.0181 ± 0.0019 L_0/F_0, a lower value than intact smooth muscle, but comparable to that reported for isolated cells. This approach can provide quantitative mechanical data in cultured cells and a potential expression system for direct modifications of the contractile apparatus. Supported by NIH HL23240 and HL22619 (RJP), and HL35808 (PdL).

SR Ca^{2+} RELEASE CHANNEL

Th-Pos312

Ca-PUMP AND Ca-RELEASE CHANNEL INTERACTION IN SKELETAL MUSCLE SARCOPLASMIC RETICULUM. **A. Chu[1] and L.G. Mészáros[2], [1]Cardiovasc. Sci. Div., Dept. Med., Baylor Coll. of Med., Houston, TX and [2]Dept. Physiol. & Endocrinol., Medical Coll. of Georgia, Augusta, GA.**

The Ca^{2+} release blocker ruthenium red in submicromolar concentrations alters the kinetic behavior of the Ca-pump (as evidenced by the measurements of phosphoenzyme formation) in sarcoplasmic reticulum vesicles that were derived from terminal cisternae (TC), which contain both the ryanodine receptor Ca-channel (RyRC, 1-5% of total protein) and Ca-pump (40-50%). On the other hand, no such effect of ruthenium red were observed in light vesicles, which were devoid of the channel, suggesting the occurrence of some sort of functional interaction involving the Ca-release channel and the Ca-pump in TC. To further test this the effects of specific Ca-pump inhibitors on the RyRC were assessed.

Using [^{3}H]-ryanodine binding at nonsaturating ligand concentration (5 nM) to monitor conformational changes of the RyRC, we found that inhibitors of the Ca-pump significantly influenced the kinetics of ryanodine binding to the channel: the amounts of ligand bound in a 15 h time period were enhanced by 100 μM vanadate (260%), 10 μM cyclopiazonic acid (230%) and 10 μM thapsigargin (220%), under conditions where the Ca-pump may be operational (1 mM MgATP and 100 μM Ca^{2+}). Since we did not find similar enhancement in [^{3}H]-ryanodine binding by the above inhibitors after extracting the pump protein from the vesicles with Triton X-100, the results presented here further support our view that some intimate communication between the channel and the pump proteins exists. [Supported by the American Heart Association, TX and GA Affiliates.]

Th-Pos313

EVIDENCE THAT CALSEQUESTRIN (CAL) AND THE RYANODINE RECEPTOR (RR) TRAVEL ALONG DISTINCT INTRACELLULAR PATHWAYS TO THE JUNCTIONAL SR. ((*C. Mason, *K.P. Campbell and °A.O. Jorgensen)) °Dept. Anatomy & Cell Biology, Univ. of Toronto, Toronto, Canada, M5S 1A8 & *HHMI, Dept. Physiol. & Biophysics, Univ. of Iowa, Iowa City, IA 52242, U.S.A.

We have used double immunofluorescence labeling and scanning laser confocal microscopy to compare the temporal appearance and spatial distribution of two junctional SR proteins (calsequestrin (CAL) and the ryanodine receptor (RR) in developing rabbit skeletal muscle cells in culture. Labeling for CAL, detected in myoblasts after 24h was first distributed in a diffuse network and later (48h) also in discrete foci present in both interior and peripheral regions of the cytosol. As development proceeded an increasing proportion of these foci were located to the peripheral regions of the developing myotubes. In contrast labeling for RR was not detected until after 70 hr in culture and was only localized in discrete foci mainly at the cell periphery. Surprisingly a majority of the foci labeled for RR and CAL did not codistribute in myoblasts and early myotubes. However as development proceeded a majority of the foci labeled for CAL and RR did codistribute at the cell periphery.

The results presented are consistent with the idea that CAL and RR both targeted for j-SR appear sequentially and following their synthesis in RER are sorted into distinct transfer vesicles prior to their assembly first into junctional complexes between j-SR and SL (peripheral couplings) and later transverse tubules (triads). °Supported by MRC of Canada°

Th-Pos314

FK BINDING PROTEIN IS ASSOCIATED WITH SKELETAL MUSCLE RYANODINE RECEPTOR FROM DIVERSE PHYLA OF VERTEBRATES

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The calcium release channel (CRC) of sarcoplasmic reticulum serves as the intracellular Ca^{2+} machinery that triggers muscle contraction. The immunophilin, FK-506 binding protein (FKBP12), was recently found to be tightly associated with the CRC of rabbit skeletal muscle terminal cisternae (*J. Biol. Chem.* 267, 1992). The aim of our studies is to assess whether FKBP is generally associated with the CRCs from diverse species. Terminal cisternae fractions were prepared from skeletal muscle from a variety of animals. ^{3}[H]FK-816 (a dihydropropyl derivative of FK-506) and high affinity ^{3}[H] ryanodine binding were used to measure the molar ratio of FKBP to CRC in mammals (rat, dog), amphibian (frog), bird (chicken) and fish (goldfish) terminal cisternae with stoichiometric ratios of approximately 4 FKBP per CRC. This ratio is comparable to that found in rabbit skeletal muscle terminal cisternae. We conclude that FKBP is generally associated with the CRC of skeletal muscle terminal cisternae in the vertebrate phyla.

Supported by NIH HL32711 (SF), Biophysical Training Grant (EF) and Muscular Dystrophy Assoc.(SF)

Th-Pos315

THE CALCIUM RELEASE CHANNEL OF CANINE HEART SARCOPLASMIC RETICULUM (SR) IS ASSOCIATED WITH TWO NOVEL FK-506 BINDING PROTEINS.

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Cardiac SR vesicles display high affinity binding sites for the immunosuppressant drug FK-506 (Kd=16 nM). The ratio of Bmax values for [^{3}H] FK-816 vs. high affinity [^{3}H] ryanodine binding sites suggest each protomer of the calcium release channel (CRC) in cardiac SR is associated with one FK-506 binding protein (FKBP). Western blot analysis of dog muscle SR preparations with antibodies raised against the N-terminal sequence of FKBP-12 detects FKBP-12 in dog skeletal muscle SR while dog cardiac SR contains immunoreactive bands of 16 and 46 Kd. Both the 16 and 46 Kd bands copurify with the cardiac CRC by sucrose gradient centrifugation, yet only the 16 Kd band remains associated with the CRC further enriched by Affigel-Heparin chromatography. Both immunoreactive bands are dissociated from the CRC by incubation with FK-506. Therefore, the stoichiometry and the interaction of these novel FKBPs with FK-506 and the cardiac CRC is similar to that which we have described for the skeletal muscle CRC and FKBP-12 (J. Biol. Chem., 268, in press, 1993). Grants NIH HL32711 and Muscular Dystrophy Association (SF).

Th-Pos316

ANIONS WHICH POTENTIATE E-C COUPLING STIMULATE THE SKELETAL MUSCLE RYANODINE RECEPTOR.

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Perchlorate is one of a group of inorganic anions which potentiate excitation-contraction coupling in skeletal muscle. We have compared the effect of perchlorate on the ryanodine receptor (RyR) of porcine sarcoplasmic reticulum (SR) with the effect of inorganic phosphate (P_i), an anion which accumulates in skeletal muscle during exercise. Perchlorate and P_i (10-20 mM) stimulated Ca^{2+} release from SR vesicles 2- to 3-fold, respectively, and increased ryanodine binding to SR vesicles 1.5-fold. Stimulation of RyR activity by both perchlorate and P_i was maximal in the presence of micromolar Ca^{2+} and was associated with an increased affinity of the channel for ryanodine. Other inorganic anions known to potentiate skeletal muscle contraction also stimulated SR Ca^{2+} release and ryanodine binding; the relative effectiveness of these anions as stimulators of ryanodine binding approximated their reported effectiveness as potentiators of muscle contraction (thiocyanate > iodide > perchlorate > nitrate). Similar effects on Ca^{2+} release and ryanodine binding activity of skeletal muscle SR were also evident in the presence of the P_i analog vanadate. However, none of the inorganic anions examined altered ryanodine binding to cardiac muscle SR. These results indicate that the skeletal muscle RyR may be a primary target at which perchlorate and other potentiating anions affect excitation-contraction coupling. Furthermore, the action of these anions on the skeletal muscle RyR resembles that of P_i, a potential endogenous regulator of this channel. Supported in part by NIH GM31382.

Th-Pos317

RT-PCR DETECTION OF RYANODINE RECEPTOR ISOFORMS AND ALLELES IN PORCINE TISSUES.

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The tissue distribution of mRNAs for ryanodine receptor (RYR) isoforms in various porcine tissues has been determined with RT-PCR. First strand cDNA was synthesized from total tissue RNA with m-MLV reverse transcriptase and random hexamer primers. PCR primers were selected to amplify a 486 bp segment in the 5' region of the skeletal or cardiac muscle RYR cDNA sequences. The skeletal muscle PCR primers amplified a skeletal muscle but not a cardiac muscle RT-PCR product, whereas the cardiac muscle PCR primers amplified a cardiac but not a skeletal muscle RT-PCR product indicating the specificity of the technique. Porcine cardiac muscle RYR RT-PCR products were identified in aorta, adrenal, kidney, and spleen, while skeletal muscle RYR RT-PCR products were identified in esophagus, small intestine, and stomach. The cardiac RYR was also identified in cerebrum and frontal lobe, while both RYR isoforms were detected in brainstem, cerebellum, temporal lobe and thalamus. The identities of the RYR RT-PCR products from skeletal and cardiac muscle, as well as from brain were confirmed by DNA sequencing. Furthermore, in brain regions containing the skeletal muscle form of the RYR, the Arg615Cys mutation was identified in malignant hyperthermia (MH) susceptible pigs. These results thus demonstrate expression of skeletal and cardiac muscle RYR isoforms in a number of porcine tissues and provide a possible explanation for a neurogenic etiology of MH. (Supported by NIH grant GM-31382).

Th-Pos318

ARE SARCOPLASMIC RETICULUM Ca^{2+} RELEASE CHANNELS IN PIGS HETEROZYGOUS FOR THE MALIGNANT HYPERTHERMIA MUTATION HOMOTETRAMERS OR HETEROTETRAMERS?

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The mutation in the porcine sarcoplasmic reticulum (SR) Ca^{2+} release channel (CRC) which is highly correlated with malignant hyperthermia susceptibility (MHS) results in abnormalities in the regulation of this channel. SR vesicles derived from pigs heterozygous for the MHS allele have Ca^{2+} release and ryanodine binding properties that are intermediate between those of homozygous MHS and normal vesicles. Thus, heterozygous pigs may have CRC populations consisting of both MHS and normal homotetramers, or heterotetrameric CRCs with properties distinct from those of the MHS or normal CRCs. To distinguish between these possibilities, 21 MHS, 20 normal, and 23 heterozygote CRC were examined in a planar lipid bilayer system. Percent open time (P_o) was 4.3 $\pm$ 0.6, 20.2 $\pm$ 4.0, and 27.8 $\pm$ 2.9 for normal, heterozygote, and MHS channels, respectively; the range of heterozygote P_o values overlapped those of the MHS and normal channels. Mean open and closed dwell times of heterozygote channels were also intermediate between MHS and normal values. However, the distribution of the heterozygote data was not consistent with a model in which the heterozygote CRCs were comprised only of MHS and normal homotetramers. Thus, the heterozygote CRC distribution must include heterotetramers with distinct kinetics. The data also indicated that more than one MHS subunit per CRC tetramer is required to produce an altered channel function detectable at the single-channel level. Supported by NIH GM31382.

Th-Pos319

VARIOUS AGONISTS SUPPORT ADAPTIVE BEHAVIOR ("QUANTAL" Ca^{2+} RELEASE) BY ISOLATED SARCOPLASMIC RETICULUM

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Intracellular Ca^{2+} release channels, including the inositol trisphosphate receptor (IP_3R) and ryanodine receptor (RyR), exhibit a unique adaptative behaviour in which multiple stepwise increases in agonist concentration lead to successive transient or partial releases of sequestered Ca^{2+}. The mechanism underlying this phenomenon is not known. Various schemes suggested to explain adaptive behaviour involve variations of the receptor's ligand sensitivity. Here we demonstrate that incremental release from RyR is not limited to one ligand (Ca^{2+}) but instead is supported by chemically diverse agonists unlikely to all interact at the same binding site. A spectrophotometric assay with KCl, KMOPS, ATP, an ATP regenerating system and antipyrylazo III was used to study Ca^{2+} release from sarcoplasmic reticulum isolated from cardiac and skeletal muscle. Addition of submaximal doses of various RyR agonists including caffeine, sulmazole, doxorubicin and sphingosine phosphorylcholine caused repeated transient releases of Ca^{2+} from apparently inactivated or desensitized stores. These results may indicate that "quantal" or incremental release is not due to a variation in channel ligand sensitivity at any one site. A mechanism which involves a common activation pathway may have to be considered instead.

Th-Pos320

CHARACTERIZATION OF CALCIUM RELEASE CHANNEL (CRC) IN FETAL, YOUNG AND ADULT RAT HEARTS.

((V. Ramesh, M. J. Kresch and D.H. Kim)) University of Connecticut Health Center, Farmington, CT 06030.

To determine whether the characteristics of CRC in rat heart sarcoplasmic reticulum undergo functional changes during development and growth, kinetics of [^{3}H]ryanodine binding were studied using whole homogenates of left and right ventricles of fetal (F; 21-22 days in gestation), young (Y; 5 weeks old) and adult (A; 12 weeks old) Sprague-Dawley rats. [^{3}H]ryanodine binding yielded similar CRC densities (pmols/mg protein) in young and adult rat hearts (LV: 0.58 ± 0.05 for Y and 0.50 ± 0.07 for A; RV: 0.59 ± 0.02 for Y and 0.61 ± 0.12 for A). However, CRC density was significantly less in F (LV: 0.22 ± 0.004; RV: 0.25 ± 0.01, P<0.05). The Ca sensitivity and cooperativity of ryanodine binding to CRC of LV and RV homogenates, measured as Ca concentration at half-maximal activation and Hill coefficient, were similar among the 3 age groups. Caffeine sensitivity, measured as caffeine-induced increase in ryanodine binding to CRC, was also similar among the 3 age groups. Our results suggest that a major functional change during postnatal growth is increased expression of CRC. (This research was supported by NIH Grant HL-33026. D.H.K. is an Established Investigator of the American Heart Association.)

Th-Pos321

PHARMACOLOGICAL DEFINITION AND MODULATION OF A NON-Ca^{2+} RELEASE CHANNEL Ca^{2+} EFFLUX PATHWAY ("LEAK") IN SKELETAL JUNCTIONAL SARCOPLASMIC RETICULUM (JSR) MEMBRANES. ((P.D. Pion, W.S. Smith, M.M. Mack and I.N. Pessah)) Dept. of Mol. Biosci., Sch. of Vet. Med., Univ. of CA, Davis, CA 95616. (Spon. by E.D. Buck).

Net flux of Ca^{2+} between the sarcoplasm and skeletal muscle JSR lumen is the vectorial sum of uptake (via the SERCA pump) and efflux rates. Addition of a saturating concentration of the SERCA pump inhibitor thapsigargin (TG) to Ca^{2+} loaded JSR vesicles results in rapid Ca^{2+} efflux despite full blockade of Ca^{2+} release channels (CRC) with 1μM ruthenium red (RR) (*FASEB J. 6(4):A1273, 1992).* The objectives of this investigation were to pharmacologically define efflux pathways which are distinct from CRC channels in JSR. ATP-dependent Ca^{2+} uptake and release from JSR vesicles (55μg protein) was monitored in the presence of pyrophosphate (7.5mM) with antipyrylazo III (250μM). The following observations were made: (1) Rapid efflux from Ca^{2+} loaded JSR was observed after addition of TG despite the presence of saturating (10X IC_{50}) concentrations of the CRC inhibitors neomycin (IC_{50} = 200nM) and FLA365 (IC_{50}=3μM) or CRC inactivating concentrations of ryanodine (500μM). (2) CRC inhibiting concentrations of ryanodine (500μM), neomycin, or FLA365 did not enhance the loading capacity of JSR. (3) However, neomycin and FLA365 applied at concentrations 2 to 3 logs greater than their IC_{50}s for CRC inhibition significantly diminished rates of TG-mediated Ca^{2+} efflux and increased 2-fold the total loading capacity of the vesicles. Neither neomycin nor FLA365 applied at similarly high concentrations significantly altered Ca-ATPase activity. We conclude, that a nonCRC ("leak") efflux pathway (defined as the Ca^{2+} efflux not blocked by ryanodine receptor saturating concentrations of RR, neomycin or FLA365, or micromolar ryanodine) accounts for a significant portion of total calcium efflux from JSR. This nonCRC efflux pathway is inhibited by high concentrations of CRC channel inhibitors, suggesting a possible relationship between the CRC and leak pathways. The identity of the "leak" pathway and means by which to selectively modulate it with pharmacological agents are currently under study.

Th-Pos322

FUNCTIONAL HYPERREACTIVE-THIOLS ON RYANODINE RECEPTOR-TRIADIN COMPLEX. ((G.H. Liu, J.J. Abramson, A.C. Zable & I.N. Pessah)) Dept. of Mol. Biosci., Sch. of Vet. Med., Univ. of California, Davis, CA 95616.

The fluorogenic sulfhydryl probe, 7-diethyl amino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) is used to characterize the functional role and location of highly reactive thiols on the ryanodine receptor/Ca^{2+} channel complex (RyR) of skeletal and cardiac junctional sarcoplasmic reticulum (SR). The receptor agonists, μM Ca^{2+} and nM ryanodine, promote a slow SR thiol-CPM reaction (k=0.0021 ± 0.0002 s^{-1}) and more than 89% of the fluorescence is associated with the 110 kDa Ca^{2+} pump. However, in the presence of RyR antagonists (mM Mg^{2+} and Ca^{2+}, or μM ryanodine), CPM rapidly forms adducts with highly reactive (hyperreactive) SR thiols (k=0.025 ± 0.002 s^{-1}). Non-reducing SDS-PAGE of CPM-labeled SR protein and Western blot analyses with anti-ryanodine or anti-triadin antibodies reveal that the hyperreactive thiols labeled by CPM when channel is closed are localized principally to the RyR and triadin. Immunoprecipitation with these antibodies further confirm this result. The results suggest that either (1) the redox state (sulfhydryl/disulfide status), or (2) the accessibility of the hyperreactive thiols on the RyR and triadin is determined by the conformational state of the channel. Covalent modification of hyperreactive thiols with nM CPM inhibits both Ca^{2+}-induced Ca^{2+} release and the gating activity of single channels reconstituted in bilayers, revealing the essential functional importance of hyperreactive thiols on channel-associated proteins. 1,4-Naphthoquinone selectively oxidizes hyperreactive thiols on RyR and triadin and releases Ca^{2+} from SR, without inhibiting Ca^{2+}-ATPase activity. The results provide direct evidence of the existence and functional role of hyperreactive thiols on the RyR and triadin in regulating the gating of SR Ca^{2+} channels.

Th-Pos323

NOVEL MODULATORS OF THE SKELETAL RYANODINE-SENSITIVE CALCIUM RELEASE CHANNEL: ROLE OF FKBP-12 IN CHANNEL MODULATION. ((M.M. Mack, T. F. Molinski & I.N. Pessah)) Dept. of Mol. Biosci., Sch of Vet. Med., Univ. of California, Davis, CA 95616.

Bastadins isolated from the sponge *Ianthella basta* selectively modulate the skeletal isoform of the ryanodine sensitive sarcoplasmic reticulum (SR) calcium channel. Bastadin 5 increases [^{3}H]ryanodine binding at all calcium concentrations in a dose-dependent manner without altering the affinity of the activator site for calcium. In addition, bastadin 5 dramatically increases the maximal binding capacity (B_{max}) for [^{3}H]ryanodine binding (1.3 ± 0.4 to 6.4 ± 2.4 pmole/mg for control and 5μM bastadin 5, respectively) and does not alter the potentiation of binding by caffeine or adenine nucleotides demonstrating the presence of a novel modulator site. [^{3}H]Ryanodine binding studies reveal that bastadin 5 decreases the inhibitory potency of Mg^{2+} (IC_{50}s are 0.45 ± 0.01 and 3.5 ± 0.9mM for control and 5μM bastadin 5, respectively) and high (> 100μM) Ca^{2+} (IC_{50}s are 0.71 ± 0.09 and 3.4 ± 0.42mM for control and 5μM bastadin 5 respectively). Bastadins induce Ca^{2+} release from actively loaded SR vesicles. This effect is antagonized by 100nM ruthenium red. Bastadin 5 inhibits loading of SR vesicles without significantly altering Ca^{2+}-Mg^{2+} ATPase activity and enhances calcium-induced calcium release. Interestingly, these effects of bastadin 5 on binding and calcium release are blocked by the immunosuppressant FK-506. FK-506 alone weakly enhances [^{3}H]ryanodine binding. However, FK-506 blocks bastadin 5-enhanced [^{3}H]ryanodine binding without altering the efficacy of caffeine or adenine nucleotides. Bastadin 5 does not alter inositol 1,4,5 trisphosphate (IP_3) mediated Ca^{2+} release from cerebellar microsomes nor enhances [^{3}H]ryanodine binding to cardiac or brain vesicles. This data suggests that bastadin 5 stereoselectively interacts with the channel associated protein FKBP12 to alter channel function.

Th-Pos324

RYANODINE-STIMULATED Ca^{2+} ACCUMULATION BY SARCOPLASMIC RETICULUM FROM NONFAILING AND FAILING HUMAN MYOCARDIUM. ((L. R. Nimer, D. H. Needleman, S. L. Hamilton, J. Krall and M. A. Movsesian)) Veterans Affairs Medical Center & University of Utah School of Medicine, Salt Lake City, UT, and Baylor College of Medicine, Houston, TX.

We examined the effect of 0.5 mM ryanodine on maximal oxalate-supported, ATP-dependent $^{45}Ca^{2+}$ accumulation (Ca^{2+} capacity) in microsomes prepared from nonfailing (n = 8) and failing (n = 10) human left ventricular myocardium. In the absence of ryanodine, Ca^{2+} capacity at 10 μM Ca^{2+} was 4.06 ± 0.58 μmol/pmol of Ca^{2+} ATPase in nonfailing hearts and 3.94 ± 0.43 μmol/pmol of Ca^{2+} ATPase in failing hearts. Incubation with 0.5 mM ryanodine increased Ca^{2+} capacity by 2.03 ± 0.40 μmol/pmol (50.2 ± 6.5 % stimulation) in nonfailing hearts but only by 0.94 ± 0.18 μmol/pmol (24.3 ± 4.1 % stimulation) in failing hearts. Ryanodine-sensitive Ca^{2+} channel density (determined by high affinity [^{3}H]ryanodine binding) was 13.3 ± 2.2 pmol/nmol of Ca^{2+} ATPase (determined by acid-stable phosphoenzyme content) in nonfailing hearts and 11.9 ± 1.6 pmol/nmol of Ca^{2+} ATPase in failing hearts. These results suggest that oxalate-supported, ATP-dependent Ca^{2+} accumulation may be decreased in microsomes derived from the junctional sarcoplasmic reticulum of failing human left ventricular myocardium because of a reduction in the efficiency of closure of sarcoplasmic reticulum Ca^{2+} channels by ryanodine.

Th-Pos325

ELECTRON CRYOMICROSCOPY OF RYANODINE–MODIFIED Ca^{2+}–RELEASE CHANNEL. ((Irina I. Serysheva, Michael B. Sherman, *Susan L. Hamilton and Wah Chiu)) Department of Biochemistry and *Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030

The Ca^{2+} release channel is responsible for rapid Ca^{2+} release from SR upon excitation–contraction coupling in muscle. The channel complex is a homotetramer composed of four subunits with M.W. ~450 kD each. The channel can be purified to homogeneity. Under physiological conditions the Ca^{2+}–release channel exists in "open" or "closed" states. We used the plant neutral alkaloid ryanodine to drive channels into the "open" state and prepared them for electron cryomicroscopy. The electron images were taken at –157°C in a JEOL1200 electron microscope at 100 kV with an electron dose ~6 electrons/Å^2. The digitized micrographs were processed with IMAGIC software (kindly provided by Dr. M. vanHeel). Over 2,000 particle images were boxed out into individual images of 80x80 pixels, translationally and rotationally aligned with respect to a reference particle image. Multivariate statistical analysis followed by classification was applied to group the particle images according to their feature similarities. Images from the same classes were summed to enhance the statistical definition of the resulting averages. The above procedure was iterated several times with different class averages as a reference image in order to separate the data more finely. The four-fold symmetry of the particle was quite apparent in the average of the predominant class. In projection, our map appears similar to that without the ryanodine shown previously by Radermacher *et al.* In order to retrieve the 3–dimensional structure of the calcium release channel, we are employing the reconstruction algorithm which requires the computational sorting of the particles at different orientations.

This work is supported by the W. M. Keck Foundation, the Muscular Dystrophy Association of America and the National Institutes of Health.

Th-Pos326

COCAINE BLOCK OF PURIFIED CARDIAC CALCIUM RELEASE CHANNELS.

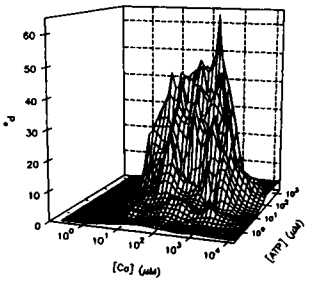
((Robert G. Tsushima and J. Andrew Wasserstrom)) Department of Medicine, Northwestern University Medical School, Chicago, IL 60611.

The toxic effects of cocaine on the heart have been shown to be the result of the inhibition of presynaptic catecholamine reuptake and the modulation of cardiac ion channels through its local anesthetic properties. Recent evidence has shown that local anesthetic agents modulate cardiac and skeletal sarcoplasmic reticular calcium release channels (ryanodine receptor). We examined the effects of cocaine on purified canine cardiac calcium release channels incorporated into planar lipid bilayers. Single channel activity was recorded under symmetrical ionic conditions (250 mM KCl, pH 7.4, pCa 5.5-4.5). Cocaine (1-10 mM) elicited a voltage-dependent block when added to either the cytoplasmic or lumenal face of the channel. At positive potentials, cocaine reduced unitary channel current and increased open-channel noise, but had no effect on the open probability. Analysis of the concentration dependence revealed that cocaine binds to a single site (Hill coefficient=1.16) with a K_d of 8.7 mM at +40 mV. The block by cocaine was well described by a two-barrier, one-well model. The dissociation rate constant at 0 mV ($K_s(0)$) was 38 mM and the effective valence ($z\delta$) was 0.9. These results demonstrate that cocaine modifies single calcium release channel kinetics in a manner similar to other local anesthetics.

Supported by the NIH, AHA Chicago Affiliate and MRC of Canada

Th-Pos327

EFFECTS OF FREE Ca, ATP AND Mg ON THE EQUILIBRIUM BEHAVIOR OF RABBIT SKELETAL MUSCLE Ca RELEASE CHANNELS IN PLANAR BILAYERS. ((J.Zhou, I.Stavrovsky,J.Ma*,E.Rios))Dept.of Physiology, Rush University, 1750 W.Harrison,Chicago,IL 60612



The activity of single channels from heavy SR reconstituted in planar bilayers, was studied by measuring Cs current in the steady state reached 1 min after changing ATP, Ca or Mg. The figure shows average Po (2 to 11 channels per point) at different free [Ca] and free [ATP] in [Mg]=0. Cuts of the surface at different [ATP] yield curves that simply scale, with no shift in [Ca]-dependence of activation or inactivation. The ATP-dependence is monotonic and ATP alone does not open the channel.

The effects of Mg on channels at 300μM free ATP include: increase in the cooperativity of activation by Ca (Hill coefficient went from 1.1 at 0 Mg to 1.7 at 100μM Mg) and, at Mg 300μM or higher, sharp reduction in maximum Po. Mg did not shift the optimum [Ca], which remained at 100μM (n=18).

Mechanistic consequences of these observations are explored in the following poster. One physiological consequence is that channels with these properties cannot conduct steady current in vivo. If they open, they will inevitably close, inactivated by Ca, and either free Mg or Mg displaced from ATP. We are grateful to M.Hosey and J.Ptasienski for help in the preparation of SR vesicles. Supported by NIH and AHA.

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Th-Pos328

ION CHANNEL PROPERTIES OF THE α AND β RYANODINE RECEPTOR ISO-FORMS CO-EXPRESSED IN AVIAN SKELETAL MUSCLE. ((A. Percival, A.J. Williams, J.A. Airey, J.L. Kenyon and J.L. Sutko)) Dept. Pharmacology, Univ. Nevada, Reno, Reno, NV 89557.

Planar lipid bilayer single channel current measurements were used to establish the ion channel properties of the α and β ryanodine receptors (RyR) which co-exist in avian skeletal muscle. The α and β RyR channels have similar conductances of 120 pS and 750 pS in 50 mM $CaCl_2$ and 210 mM KCl, respectively, but they differ markedly in their gating behaviors and in their sensitivities to the activating effects of Ca^{2+} and ATP. The αRyR channel has short openings (τ_O = 0.11 msec) and assumes two quasi-stable modes of gating. In a low activity mode this channel is insensitive to Ca^{2+} in the absence of ATP, but is activated by Ca^{2+} in the presence of ATP. In a high activity mode, the αRyR channel is sensitive to Ca^{2+} in both the absence and presence of ATP. The βRyR channel has longer openings (τ_O = 0.92 and 5.55 msec) and is more sensitive to activation by Ca^{2+} in the absence of ATP than either of the gating modes exhibited by the αRyR channel. The α and β RyR channels also differ in their sensitivities to the inhibitory effects of high concentrations of Ca^{2+} and this is affected by the presence of ATP. Both the α and β RyR channels are inactivated by positive voltages. Thus, these findings indicate that the α and β RyR channels have different properties which may permit these proteins to assume different roles in coupling excitation to SR calcium release in avian muscle.

Th-Pos329

EFFECT OF TEMPERATURE ON FUNCTION OF RYRI CHANNEL IN A LIPID BILAYER. ((T.E. Nelson and M. Lin)) Dept. of Anesthesia Bowman Gray School of Medicine, Winston-Salem, NC 27157-1009

Previously we reported (1) the effects of temperature on (a) development of the anesthetic-induced malignant hyperthermia (MH) syndrome in pigs, (b) *in vitro* MH muscle contracture to halothane, and (c) on abnormal Ca^{++} release from isolated SR membrane vesicles. In the present study, we report preliminary data from investigations of temperature effects on single, wild-type (normal) RYRI calcium channels in a planar lipid (POPE: POPC, 70:30) bilayer. The bilayer chamber was surrounded by a brass block though which water was circulated for temperature control. Seven different channels were recorded at different temperatures ranging from 22-37°C. Voltage-induced capacity transients in the bilayer were measured at various temperatures in the absence of protein to assess temperature effects on the bilayer. The recorded channels had average amplitude of 19.7 pA and two open and two closed states (τo_1=0.31 ms, τo_2=2.3 ms, τc_1=3.7 ms, τ_2=16.46). These variables did not significantly change with temperatures ranging from 22-37°C. The open state probability of the channels changed very little from 22 to 32°C while above 32°C the Po increased with a peak near 34°C. Bilayer capacitance increased linearly by 0.36 pF/°C until about 36°C where a sharp transition occurred (*ie*, 4 pF/°C) with a peak at 38°C. The change in Po with temperature is not explained by these temperature effects on the bilayer.

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Th-Pos330

ISOLATION AND CHARACTERIZATION OF RYANODINE RECEPTOR TOXINS FROM *HELODERMA HORRIDUM* (MEXICAN BEADED LIZARD) VENOM. ((J. Morrissette, R. El-Hayek, L. Possani^ and R. Coronado)) Dept. of Physiology, Univ. of Wisconsin, Madison, WI. 53706. ^Departmento de Bioquimica, Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico.

Helothermine, a 25 kDa toxin purified from the venom of *Heloderma horridum* was previously found to induce lethargy, partial paralysis and lowering of body temperature when injected into mice. Helothermine had no effect on voltage dependent Na^+,K^+ or Ca^{2+} channels. (Toxicon, 28:299-309, 1990). We now show that the physiological effects may be due to inhibition of the ryanodine receptor Ca^{2+} release channel. Helothermine inhibited [^{3}H]ryanodine binding to HSR vesicles from rabbit skeletal muscle with an IC_{50}~100 nM. Likewise, μM concentrations decreased the open probability of Ca^{2+} release channels incorporated in lipid bilayers. We also fractionated the whole *Heloderma* venom by reverse phase HPLC and found two other distinct peptides, helo II (15 kDa) and helo III (80 kDa), that inhibited Ca^{2+} release channels incorporated into lipid bilayers. These two toxins also inhibited [^{3}H]ryanodine binding to HSR vesicles with an IC_{50} of ~66 nM and ~100 nM respectively. Amino acid sequence analysis revealed that helo II and helo III shared 100 % N-terminal homology with phospholipase A_2 and exendin-3 respectively: two peptides that were previously purified from *Heloderma* venom. Supported by NIH, AHA, and MDA.

Th-Pos331

ISOLATION AND CHARACTERIZATION OF RYANOTOXIN, A PEPTIDE FROM THE SCORPION *BUTHOTUS JUDIACUS* WHICH MIMICS THE ACTION OF RYANODINE ON CA^{2+} RELEASE CHANNELS. ((J. Morrissette and R. Coronado)) Dept. of Physiology, Univ. of Wisconsin, Madison, WI 53706.

We purified a ~10 kDa toxin (ryanotoxin) from the venom of *Buthotus judiacus* by reverse phase HPLC. Ryanotoxin (1 μM) stimulated [^{3}H]ryanodine binding to rabbit skeletal muscle HSR vesicles and induced a subconducting state in Ca^{2+} release channels incorporated into lipid bilayers. I-V analysis indicated that the ryanotoxin modified channel had a reduced unitary conductance in 250 mM cis Cs methanesulfonate 50 mM trans Cs methanesulfonate of 163±12 pS corresponding to a 40% reduction of the 387±35 pS unitary conductance of the unmodified channel. Under the identical recording conditions, 10 μM ryanodine induced a 174±26 pS subconducting state corresponding to a similar ~40% reduction of the 405±14 pS initial unitary conductance. The P_o of the subconducting state was ~0.4 and ~1.0 in the ryanotoxin and ryanodine modified channel respectively, whereas the P_o of the control channel was ~0.1 in both cases. In addition, ryanotoxin stimulated a ruthenium red sensitive Ca^{2+} release from actively loaded HSR vesicles as measured by fura-2. These results indicate that ryanotoxin and ryanodine may share a common binding site on the receptor. Supported by NIH, AHA and MDA.

Th-Pos332

4-AMINOPYRIDINE ACTIVATES THE SARCOPLASMIC RETICULUM CALCIUM RELEASE CHANNEL/RYANODINE RECEPTOR OF SKELETAL MUSCLE ((Le Xu and Gerhard Meissner)) Department of Biochemistry and Biophysics, University of North Carolina , Chapel Hill, NC 27599-7260.

4-Aminopyridine, a K^+ channel blocker and clinical drug, has been found to interact with the sarcoplasmic reticulum (SR) ryanodine receptor of rabbit skeletal muscle in [^{3}H]ryanodine binding and single channel measurements. Ryanodine binding was assayed in the presence of 0.15 M KCl, 25 μM free Ca^{2+} , 5 nM [^{3}H]ryanodine and various concentrations of 4-aminopyridine. 4-Aminopyridine (50 mM) resulted in a 2.4, 1.6, and 1.1 fold increase of [^{3}H] ryanodine binding at pH 6.4, 7.0, and 7.6, respectively. The effect of 4-aminopyridine on the Ca^{2+} release channel of skeletal muscle was also examined by reconstituting the Chaps-solubilized, purified 30 S channel complex into planar lipid bilayers in a symmetric 0.25 M KCl medium at pH 7.0. 4-Aminopyridine (10 mM) increased channel open probability 3 to 10 fold but did not change the conductance of the channel. Time analysis showed that 4-aminopyridine increased open time constants and decreased closed time constants.

Th-Pos333

EFFECT OF SR LUMENAL CA^{++} ON THE OPEN PROBABILITY OF THE SKELETAL MUSCLE RYANODINE RECEPTOR/ CA^{++} RELEASE CHANNEL ((Ashutosh Tripathy and Gerhard Meissner)) Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27599-7260. (Spon. by Jogin R. Wu).

It is well documented that the sarcoplasmic reticulum Ca^{++} release channel is regulated by μM cis (cytoplasmic) Ca^{++}. Studies involving the effects of trans Ca^{++} have been contradictory, though the first recordings of the release channel were made in 55 mM trans Ca^{++}. We have, therefore, reexamined the role of trans Ca^{++} on the open probability (P_O) of the release channel. Purified ryanodine receptors were reconstituted into liposomes, which were fused with planar lipid bilayer membranes in the presence of a salt gradient. The conductance and P_O were measured in symmetrical 200 mM KCl at negative 40 mV applied potential. In reasonable agreement with the results of vesicle-ion flux studies, our bilayer studies consistently showed peak activation at free cis [Ca^{++}] of 50-100 μM. The trans Ca^{++} was varied from 50 μM to 10 mM. In most of the experiments an increase in P_O was observed as the trans [Ca^{++}] was raised. The P_O at 10 mM trans Ca^{++} increased by 60% from the corresponding value at 50 μM trans Ca^{++}. Increase of trans Ca^{++} resulted in a decrease in the K^+ conductance of the release channel.

Th-Pos334

CHLORIDE CHANNELS LEAKY TO Ca^{2+} IN SARCOPLASMIC RETICULUM OF RABBIT SKELETAL MUSCLE. ((Roberto Coronado, Manana Sukhareva and Jeffery Morrissette)) Dept. of Physiology, Univ. of Wisconsin Medical School, Madison, WI 53706.

We described a new pathway for Ca^{2+} efflux from SR which accounts for Cl^- induced Ca^{2+} release (Sukhareva and Coronado, 1994. B.J. Abstract). Here we investigated if the ionic mechanism involved a Cl^--dependence of the ryanodine receptor or was due to a Ca^{2+} leak through SR Cl^- channels. Open probability of ryanodine receptors was 0.1±0.08 (n=12) in low cis Cl^- (240 mM CsMethanesulfonate, 10 mM CsCl, pCa 6) and was 0.106±0.05 (n=12) in high Cl^- cis (250 mM CsCl, pCa 6). When SR was equilibrated in low and high Cl^-, $^{45}Ca^{2+}$ released at t=50 ms was 29.5±3 nmoles/mg (n=4) and 57.9±0.3 nmoles/mg (n=4), respectively. Thus ryanodine receptor channel activity did not change with Cl^- although Cl^- induced Ca^{2+} release could be demonstrated. A significant Ca^{2+} permeability was detected in a SR Cl^- channel by reversal potentials in TrisCl and $CaCl_2$ gradients. The single channel conductance was ~100 pS in cis/tras 450/50 mM TrisCl and the permeability ratios were PCl:PTris:PCa=1:0.5:0.3. Cl^- induced SR Ca^{2+} release may originate in a low-selectivity SR channel which is blocked by the large anions used to replace Cl^-, typically gluconate$^-$, and becomes unblocked when SR es exposed to Cl^- and the organic anion is removed. Supported by NIH, MDA, and AHA.,

Th-Pos335

A RE-EVALUATION OF Cl^- INDUCED Ca^{2+} RELEASE IN THE SARCOPLASMIC RETICULUM OF RABBIT SKELETAL MUSCLE. ((M. Sukhareva and R.Coronado)) Dept. of Physiology, Univ. of Wisconsin Medical School, Madison, WI 53706.

The rate of $^{45}Ca^{2+}$ release from passively-loaded junctional SR was significantly higher when 150 mM Cl^- was present on the outside-only, inside-only or both sides of the SR membrane. Cl^- could not be substituted by Br^-, I^-, Acetate$^-$, Gluconate$^-$, HEPES$^-$ or Methanesulfonate$^-$. The stimulation of release by ATP (5 mM total) and caffeine (20 mM) and the inhibition of release by procaine (10 mM) and Mg^{2+} (0.8 mM free) were the largest in 150 mM Cl^--free solution (K-Gluconate), intermediate in KCl, and notoriously poor or absent in Cl^--containing univalent salts with organic cation (Choline-Cl or TRIS-Cl) . Ruthenium red (RR) inhibited Ca^{2+} release in all solutions. However a higher RR concentration (4 fold) was needed to block Ca^{2+} release in Choline-Cl than in K-Gluconate. Cl^--induced Ca^{2+} release was enriched in junctional SR sedimenting in 36% sucrose (w/v) and was not present in light SR sedimenting in 18% sucrose (w/v). Cl^--induced Ca^{2+} release was not blocked by Ca^{2+} pump inhibitors, was not affected by K-valinomycin, was not due to a Ca^{2+}-Ca^{2+} exchange reaction, and was not due to an osmoticaly-driven influx of water. We suggest that, Cl^- induces a RR-sensitive Ca^{2+} release pathway or channel with a ligand-dependence that is different from that of ryanodine receptors. This pathway operates in paralell with that mediated by ryanodine receptors. (Supported by NIH,MDA and AHA).

Th-Pos336

PARTIAL cDNA SEQUENCE OF LOBSTER SKELETAL MUSCLE RYANODINE RECEPTOR/Ca^{2+} RELEASE CHANNEL. ((H. Xiong, L. Hines, W. Liu, X. Feng, G. Meissner)) Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, NC 27599-7260

We have cloned and sequenced the putative membrane-spanning region of the lobster skeletal muscle Ca^{2+} release channel/ryanodine receptor (RYR) and compared the deduced amino acid sequence with the mammalian RYR sequences. The lobster and mammalian RYRs have similar ion conducting properties but exhibit a distinctly different pattern of regulation by endogenous and exogenous effector molecules. The isolated lobster cDNA sequence encodes 1511 of about 5000 amino acids of the mammalian RYR polypeptides. The deduced peptide sequence shows 72% homology to rabbit brain and cardiac RyR, and 71% homology to rabbit skeletal muscle RYR. The C-terminal last 400 amino acids are the most conserved, exhibiting 82% homology (65% identity) with the mammalian RYRs. Analysis of potential ligand binding sites indicated the presence of one nucleotide binding consensus sequence GXGXXG which is not conserved in the mammalian sequences, and two EF-hand Ca^{2+} binding sites which are partially conserved in the mammalian counterparts.

Th-Pos337

IDENTIFICATION OF CALMODULIN, Ca^{2+} AND RUTHENIUM RED BINDING DOMAINS IN THE Ca^{2+} RELEASE CHANNEL (RYANODINE RECEPTOR) OF RABBIT SKELETAL MUSCLE SARCOPLASMIC RETICULUM. **((S. R. Wayne Chen and David H. MacLennan)) Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6.**

A series of trpE fusion proteins containing fragments of the skeletal muscle ryanodine receptor were constructed and expressed in bacteria. These fusion proteins which cover about 90% of the linear sequence of the ryanodine receptor were used to identify calmodulin (CaM), Ca^{2+} and ruthenium binding regions in the ryanodine receptor through [^{125}I]-CaM, $^{45}Ca^{2+}$ and ruthenium red overlay procedures. Up to five major and minor CaM binding domains could be detected in the skeletal muscle ryanodine receptor. Major CaM binding domains were localized to short sequences 6b3, 11b1, and 13b2, between amino acid residues 2063 and 2091, 3611 and 3642, and 4303 and 4328. Minor CaM binding domains were identified in regions 4b, 9b, and 10a, between amino acid residues 921 and 1173, 2804 and 2930, and 2961 and 3084. All of these CaM binding domains, except for 6b3 and 4b regions, correspond to all or part of a predicted CaM binding domain in the skeletal or cardiac ryanodine receptor. Major $^{45}Ca^{2+}$ and ruthenium red binding regions were localized to the NH_2- and COOH-terminal regions and to regions 6b and 12b, between amino acid residues 1861 and 2094 and 3657 and 3776. The identification of [^{125}I]-CaM, $^{45}Ca^{2+}$ and ruthenium red binding domains revealed that major CaM, Ca^{2+} and ruthenium red binding domains are colocalized in the ryanodine receptor sequence. (Supported by the Medical Research Council of Canada).

Th-Pos338

MODULATION OF THE RYANODINE-RECEPTOR CHANNEL IN SKINNED FIBERS FROM SLOW- AND FAST-TWITCH SKELETAL MUSCLES. ((J.Y. Su and Y.I. Chang)) Dept. of Anesthesiology, University of Washington, Seattle, WA 98195

Ryanodine is shown to have high specificity and affinity to a receptor in the SR of striated muscle that is modulated by caffeine, Ca^{2+}, and Mg^{2+}. These modulators are also found to affect depression of caffeine-induced tension transients by ryanodine in skinned myocardial fibers (RYA-depression) (Pflügers Arch 421:1-6,1992). This study was performed to examine the influence of the modulators on RYA-depression in skinned fibers from adductor magnus (AM) and soleus (SL) of the rabbit. RYA-depression, at IC_{50} ryanodine (AM=3 μM, SL=10 μM), was a function of [caffeine], or [Ca^{2+}] which was potentiated by caffeine, and an inverse function of [Mg^{2+}]. At pCa>8.0 plus caffeine, a dose-dependent RYA-depression was observed, indicating Ca^{2+}-independency. At IC_{50} ryanodine, RYA-depression was significantly less sensitive in AM than in SL to the presence of pCai (pCa_{50} 4.75 vs. 5.44), or [caffeine]i (Caffeine$_{50}$ 12.5 mM vs. 7.3 mM). However no difference between these muscle types was observed with 25 mM caffeine plus pCai or plus pMgi (pCa_{50} 6.93 vs. 6.88; pMg_{50} 3.6 vs. 3.6). Thus, AM is more sensitive than SL to ryanodine with respect to [caffeine], pCai, and pMgi. The RYA-depression is modulated in both types of skeletal muscle as well as cardiac muscle. (Supported by NIH GM48243)

Th-Pos339

SURAMIN-INDUCED Ca^{2+} RELEASE FROM SARCOPLASMIC RETICULUM VESICLES: EFFECTS ON THE RELEASE CHANNEL AND THE Ca^{2+} PUMP. ((J.T. Emmick, S. Kwon, K.R. Bidasee, Y. Liang, K.T. Besch, and H. R. Besch, Jr.)) Dept. of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202.

Suramin is a polysulfonated naphthylurea originally developed to treat trypanosomiasis that has recently gained recognition for its anti-cancer activity. Suramin is also an antagonist of ATP at the P_2 purinergic receptor. In the present study, suramin is shown to evoke Ca^{2+} release from skeletal sarcoplasmic reticulum (SR) vesicles in a concentration-dependent manner. This effect is specific to junctional SR vesicles, showing that the actions of suramin involve a component unique to the terminal cisternae. The effects of suramin are not blocked by DTT, indicating that oxidation of sulfhydryl groups is not requisite for suramin actions. [^{3}H]Ryanodine binding increases significantly (1.4-fold) in the presence of suramin; this increase is similar to the 1.3-fold enhancement observed in the presence of AMP-PCP under identical binding conditions. Suramin has previously been shown to interact with other adenine nucleotide binding sites including the SR Ca^{2+} pump and Na^+,K^+-ATPase. These observations suggest that suramin induces Ca^{2+} release from SR vesicles by an agonist action at the adenine nucleotide domain of the SR Ca^{2+} release channel and by an inhibitory action at the Ca^{2+} pump. Supported in part by the Showalter Trust Fund.

Th-Pos340

SINGLE CHANNEL CONDUCTANCE OF THE SKELETAL MUSCLE RYANODINE RECEPTOR IS MODULATED BY SPHINGOSINE. ((N. Markus, G. Harris, R. Betto, G. Salviati and R. Sabbadini))
Dept. of Biology San Diego State University, San Diego CA 92182. Dipartimento Scienze Biomediche Sperimentali, University of Padova Italy. (Spon. by R. Sabbadini)

We have previously shown that the endogenous lipid second messenger, sphingosine (SPH), can modulate calcium (Ca) release from and [3H]-ryanodine binding to skeletal muscle sarcoplasmic reticulum (SR) membranes (Sabbadini et al., JBC 267:15475-15484, 1992). However, direct actions of SPH on the SR ryanodine receptor (RyR) calcium channel were inferred but not specifically demonstrated. In this study, we examined the effects of SPH and its analogues on single channel conductances of the skeletal muscle RyR reconstituted into planar lipid bilayers. We demonstrate that SPH has dual actions on single channel conductance of the receptor - at low concentrations (μM), the open probability (Po) is reduced to near-zero, while at comparatively high levels of SPH (50 μM), the Po is increased in a dose-dependent manner. Control experiments using other sphingolipid analogues demonstrate that the inhibitory effect of SPH on the channel conductance is specific and not mediated by nonspecific lipid effects. These data show that SPH acts directly on the SR RyR and confirm our previous Ca release and ryanodine binding measurements. Supported by the MDA, AHA Calif. Affiliate, NSF, CNR, Telethon-Italy and Minestero della Pubblica Istruzione of Italy.

Th-Pos341

MODULATION OF SKELETAL MUSCLE Ca^{2+} RELEASE CHANNEL ACTIVITY BY SPHINGOSINE. ((D.H. Needleman, A.B. Seryshev, G.J. Schroepfer, Jr.[1], S.L. Hamilton)) Dept. of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030; [1]Depts. of Biochemistry/Cell Biology and Chemistry, Rice University, Houston, TX 77251. (Sponsored by Steen E. Pedersen)

D-Erythro-sphingosine (SPH) is reported to be a putative second messenger in a number of systems. In the present study, we examined the effect of SPH and related compounds on the calcium release channel (ryanodine receptor) in rabbit skeletal muscle membranes and in purified preparations. SPH inhibited [^{3}H]ryanodine binding to SR membranes in a dose-dependent manner (IC_{50}=12 μM at 5 nM [^{3}H]ryanodine)similar to results published by others using commercial sphingosine (Sabbadini, R.A. *et al.* (1992), *JBC* 267:15475-15484). SPH also inhibited [^{3}H]ryanodine binding to the purified receptor. When the [^{3}H]ryanodine concentration was raised to 250 nM, SPH did not completely inhibit ryanodine binding (maximum inhibition ~25% at 100 μM sphingosine). SPH (10 μM) greatly increased the rate of dissociation of bound [^{3}H]ryanodine from SR membranes while 100 μM ryanodine slowed the dissociation of bound [^{3}H]ryanodine. The rate of dissociation of bound [^{3}H]ryanodine was higher in the presence of 10 μM SPH and 100 μM ryanodine than in the presence of 100 μM ryanodine alone. SPH had no apparent effect on the association rate of [^{3}H]ryanodine to the high affinity site in SR membranes. The effect of SPH on the ryanodine receptor occurred at concentrations lower than those reported for a number of the effects of SPH and close to the reported levels of sphingosine in human cells. L-Threo-sphingosine and D-erythro-dihydro-sphingosine also inhibited [^{3}H]ryanodine binding to SR membranes (IC_{50}=16 μM and 30 μM, respectively) where as sphingophosphorylcholine and sphingomyelin had no significant effect on [^{3}H]ryanodine binding. Our data are consistent with a model in which D-erythro-sphingosine is a noncompetitive inhibitor of ryanodine binding.

Th-Pos342

EFFECT OF MODULATORS OF THE Ca^{2+} RELEASE CHANNEL ON THE ABILITY OF RYANODINE TO PROTECT ITS BINDING SITE FROM TRYPTIC DIGESTION. ((J-P. Wang, K. Slavik, A. Seryshev, D. Needleman, C. Callaway, C. Cantu III, and S.L. Hamilton)) Baylor College of Medicine, Houston, TX 77030.

Treatment of the membrane bound form of the Ca^{2+} release channel with trypsin results in a rapid loss in the channel's ability to bind [^{3}H]ryanodine. In planar lipid bilayers mild tryptic digestion activates the channel in the absence of ryanodine. Tryptic digestion of the ryanodine modified channel reduces the conductance of the channel. As reported by Meissner *et al.* (*J. Biol. Chem.* 264:1715-1722, 1989) the occupation of sites by ryanodine partially protects the site from tryptic digestion. In addition, the occupation of low affinity sites by ryanodine offers almost complete protection against loss of [^{3}H]ryanodine bound to the high affinity site upon proteolysis. This effect is not observed with ruthenium red. After proteolysis in the absence of any of the inhibitors, ryanodine can still slow the dissociation of [^{3}H]ryanodine bound to the high affinity site. Although ruthenium red slows the dissociation of [^{3}H]ryanodine bound to the high affinity site in control membranes, this effect is rapidly lost with proteolysis. Sphingosine is also an inhibitor of [^{3}H]ryanodine binding, but instead of slowing dissociation of [^{3}H]ryanodine this compound enhances the rate at which [^{3}H]ryanodine dissociates from its binding site. This effect of sphingosine is not lost upon proteolysis. Since the proteolytic digestion of the membrane bound receptor converts the receptor to a 10S species made up of only the last 562 amino acids at the carboxy terminus we conclude that the sites at which ryanodine and sphingosine interact with the receptor are on the carboxy terminal portion of the protein. (Supported by grants from the National Institutes of Health and the Muscular Dystrophy Association of America.)

Th-Pos343

FAST ACTIVATION OF SINGLE SKELETAL MUSCLE RYANODINE RECEPTOR CHANNELS BY FLASH PHOTOLYSIS OF CAGED-Ca^{2+}. ((Sandor Gyorke, Patricio Velez, Benjamin Suarez-Isla*, and Michael Fill))
Dept. Physiology & Biophysics, Univ. Texas Medical Branch, Galveston, TX 77555-0641 and the *Dept de Fisiologia y Biofisica, Univ. Chile, Fac. Med., Casilla 70005, Santiago 7, CHILE.

Single ryanodine-sensitive SR Ca^{2+} release channels isolated from rabbit skeletal and canine cardiac muscle were reconstituted in planar lipid bilayers. Channel activity was measured in simple solutions (no ATP or Mg^{2+}) with 250 mM symmetrical Cs^+ as charge carrier. A laser flash was used to photolyze caged-Ca^{2+} (DM-nitrophen) in a small volume directly in front of the bilayer. The free [Ca^{2+}] in this small volume and in the bulk solution was monitored with Ca^{2+} electrodes. This setup allowed fast, calibrated free [Ca^{2+}] stimuli to be repetitively applied to single SR Ca^{2+} release channels. A standard photolytically-induced free [Ca^{2+}] step (pCa 7-6) was applied to both the cardiac and skeletal release channels. The rate of channel activation was determined by fitting a single exponential to ensemble currents generated from at least 50 single channel sweeps. The time constant of activation was 1.43 $\pm$ 0.65 (mean$\pm$S.D.; n=5) for cardiac and 1.28 $\pm$ 0.61 (n=5) for skeletal channels. We present a method for defining the fast kinetics of Ca^{2+} regulation of single SR Ca^{2+} release channels and shows that skeletal release channel activation is sufficiently fast to be consistent with a role for CICR in skeletal muscle E-C coupling. Supported by NIH R29AR41197.

Th-Poe344

BARIUM BLOCKADE OF CARDIAC RYANODINE RECEPTOR CHANNELS. ((Patricio Velez, Qiang Tu, Xuande Li, and Michael Fill)) Dept. Physiology & Biophysics, Univ. Texas Medical Branch, Galveston, TX 77555-0641

Single ryanodine-sensitive sarcoplasmic reticulum (SR) Ca^{2+} release channels were isolated from canine cardiac muscle. Channels were reconstituted in planar lipid bilayers by fusion of SR microsomes. Lipid bilayers were cast from an 8:2 mixture of phosphatidylethanolamine and phosphatidylecholine (in decane). Standard recording solutions contained (in mM): 200 $CsCH_3SO_3$, 20 CsHEPES (pH 7.4), 0.04 $CaCl_2$. Application of Ba^{2+} to either side of the channel attenuated conduction. Single channel events were clearly resolved in [Ba^{2+}]'s greater than 10 mM. Conduction attenuation was due to Ba^{2+}/Cs^+ competition as charge carrier. Application of 5 mM Ba^{2+} to the lumenal side of the channel did not significantly change open probability (Po). However, application of 5 mM Ba^{2+} or Sr^{2+} to the cytoplasmic side of the channel reversibly reduced Po to near zero regardless of whether the charge carrier was Cs^+ or Ba^{+2}. In 2 mM Ba^{2+} (cytoplasmic side), the reduction in Po was reversed by addition of Ca^{2+} (0.1 → 10 mM). Channel sidedness was determined by the sidedness of ATP action. These data indicate that divalent cations can effectively compete at the Ca^{2+} activation site on the cytoplasmic side of the channel. This leads to the possibility that physiological levels of Mg^{2+} inhibit channel activity by competing with Ca^{+2} for the Ca^{2+} activation site. Supported by NIH grant R29AR41197.

Th-Poe345

SLOWING OF SINGLE CARDIAC MUSCLE RYANODINE RECEPTOR CHANNEL GATING BY VISCOUS SOLUTIONS. ((Qiang Tu, Patricio Velez, Malcolm Brodwick, and Michael Fill)) Dept. Physiology & Biophysics, Univ. Texas Medical Branch, Galveston, TX 77555-0641

Single ryanodine-sensitive sarcoplasmic reticulum (SR) Ca^{2+} release channels were isolated from canine cardiac muscle and reconstituted in planar lipid bilayers. Standard recording solutions contained (in mM): 200 $CsCH_3SO_3$, 10 CsHEPES (pH 7.4), 0.04 $CaCl_2$. Solution viscosity was measured as a function of conductivity and fall time of a 3 mm diameter etched glass ball through a solution column. Addition of 1 M sucrose (symmetrical) decreased Cs^+ conduction (gCs) and dramatically slowed single channel gating. The decrease in gCs could be reversed if additional Cs^+ was added. However, the slowing of channel gating was not due to osmolarity or changes in the diffusion coefficients of Ca^{2+} or Cs^+. Sucrose action on gating did not saturate and occurred only when sucrose was applied to the cytoplasmic side of the channel. Experiments with other saccharides (glucose, raffinose, stachyose, & dextran) indicate that the slowing of channel gating is directly proportional to microscopic properties of viscosity. Analysis under control conditions defined 3 open and 3 closed time constants. Viscosity shifted open and closed time constant in parallel. The shift was greatest for the slowest time constants. These data support the hypothesis that physical restriction of protein motion (by viscosity) slows channel gating. Supported by NIH grant R29AR41197.

Th-Poe346

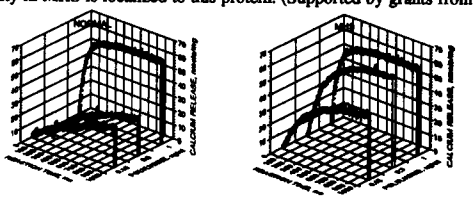
IMPERATOXIN A, A SELECTIVE ACTIVATOR OF SKELETAL RYANODINE RECEPTORS (RYR), DISTINGUISHES BETWEEN *α* AND ß RYR ISOFORMS IN NON-MAMMALIAN MUSCLE. ((H. H. Valdivia[1], O. Fuentes[1] and B. Block[2])). [1]University of Maryland at Baltimore and [2]University of Chicago.

Immunological results have led to the finding that the skeletal muscle of fish, amphibians and birds express two isoforms (*α* and ß) of the SR calcium release channel (RYR). This is in contrast to mammalian skeletal muscles, which express only one RYR isoform. Although immunological cross-reactivity of the *α* and ß RYR with mammalian skeletal and cardiac RYR, respectively, suggests structural similarities, a functional correlation between isoforms has not been demonstrated. Imperatoxin A ($IpTx_A$), a peptide component of *P. imperator* scorpion venom, selectively activates RYR of mammalian skeletal muscle (Valdivia, et al. *PNAS 89:*12185). In rabbit skeletal muscle, [^{125}I]$IpTx_A$ binds to a single class of receptors with maximal density (B_{max}) similar to that of [^{3}H]ryanodine but bovine cardiac muscle lacks [^{125}I]$IpTx_A$ receptors. We used $IpTx_A$ in non-mammalian muscles to identify the proportion of skeletal-type RYR isoforms and assess their functional properties. The B_{max} of RYRs in non-mammalian muscle microsomes was determined by constructing Scatchard plots of [^{3}H]ryanodine binding. Concentration-response curves for $IpTx_A$-activation of [^{3}H]ryanodine binding then revealed the fraction of skeletal-like RYR present in the sample. This number was compared with the ratio $B_{max}IpTx_A/B_{max}Ryd$ (B_{max} for [^{125}I]$IpTx_A$ binding divided by B_{max} for [^{3}H]ryanodine binding). Both procedures yielded similar results and indicated that almost half of RYRs from skeletal muscles of frog, turtle and fish (immunologically *α*, ß), and the majority of RYRs from skeletal muscles of rabbit and lizard and the swimmbladder fish muscle (*α* only) are sensitive to $IpTx_A$. Supp. by NIH, AHA.

Th-Poe347

POLYLYSINE-INDUCED CALCIUM RELEASE IN SARCOPLASMIC RETICULUM ISOLATED FROM NORMAL AND MALIGNANT HYPERTHERMIA-SUSCEPTIBLE PIG SKELETAL MUSCLE. ((R. El-Hayek[1], M. Yano[1], B. Antoniu[1], C. F. Louis[2], and N. Ikemoto[1,3]). [1]Boston Biomed. Res. Inst.; [2]Dept. Vet. Biol., Univ. of Minnesota; [3]Dept. Neurol. Harvard Med. Sch., Boston, MA.

The main abnormality in pig malignant hyperthermia-susceptible (MHS) muscle, is an altered gating of the SR calcium channel. We investigated the dose-dependence of SR calcium release using polylysine. As shown in the figures, at lower concentrations of polylysine (0.15-0.3 µg/ml) both the magnitude and the rate of induced calcium release are significantly higher in the MHS SR than normal SR. At 0.3 µg/ml polylysine, for example, the initial rate of calcium release from the MHS SR is about 5 times larger than that from normal SR, which is significantly higher than reported values in the literature (≤ 2). Since in this concentration range, polylysine binds specifically to the ryanodine receptor, these findings further support that the observed abnormality in MHS is localized to this protein. (Supported by grants from NIH and MDA).



Th-Poe348

BLOCKAGE OF THE T-TUBULE→SR COMMUNICATION BY NEOMYCIN. ((M. Yano[1], R. El-Hayek[1], B. Antoniu[1], and N. Ikemoto[1,2]). 1, Boston Biomed. Res. Inst.; 2, Dept. Neurology, Harvard Med. Sch., Boston, MA. (Sponsored by T. Scott).

Neomycin, like ruthenium red, inhibits drug- or Ca^{2+}-induced Ca^{2+} release from SR at micromolar concentrations, and is regarded as one of the most potent Ca^{2+} release channel blockers. In such a concentration range, neomycin binds specifically to the ryanodine receptor (RyR)/Ca^{2+} release channel protein. We now report that at even much lower concentrations neomycin produces specific inhibition of T-tubule-mediated SR Ca^{2+} release. We have investigated the effect of various concentrations of neomycin on SR Ca^{2+} release induced by two distinctly different methods: (a) polylysine that exerts direct stimulation by binding specifically to the RyR, and (b) stimulation *via* T-tubule depolarization generated by ionic replacement procedures. It was found that the inhibitory concentration range for b is at least one order of magnitude lower than that for a. Thus, 0.1 µM neomycin produced almost complete inhibition of depolarization-induced Ca^{2+} release with virtually no significant effect on polylysine-induced release. These results suggest that there are two types of neomycin binding sites. One with a lower affinity ($K \approx 1.0 \times 10^6\ M^{-1}$) located in the RyR is involved in blocking the Ca^{2+} channel, while the other has a higher affinity ($K \approx 4.3 \times 10^7\ M^{-1}$) and is involved in the blockage of the T-tubule mediated stimulation. Effort is currently made to identify the high affinity neomycin binding site(s). (Supported by grants from NIH and MDA).

Th-Poe349

RYANOID BINDING ISOTHERM HAS TWO DISTINCT COMPONENTS. ((H.R. Besch, Jr., K.R. Bidasee, S. Kwon, R.A. Humerickhouse and J.T. Emmick)) Dept. of Pharmacol. and Toxicol., Indiana Univ. Sch. of Med., Indianapolis, IN 46202.

The neutral alkaloid ryanodine has been extensively used as a tool to study the role of intracellular calcium in the excitation-contraction coupling processes. However, to date, little is certain about the molecular basis of the interactions that prompt this compound to activate and then deactivate the sarcoplasmic reticulum calcium-release channel. In prior attempts to elucidate the mechanism responsible for this duality, we employed a photo-activatable, radio-iodinated derivative of ryanodine, C_{10}-O_{eq} N-(4-azido-5 ^{125}iodo salicyloyl)-ß-alanyl-ß-alanyl ryanodine. Unfortunately, the multiple-step synthesis of this probe rendered its specific activity low (1400mCi/mmol) and vitiated its ability as a unique radioligand in equilibrium binding affinity assays. Here we describe the synthesis and use of an alternate probe with a 1000-fold higher specific activity, C_{10}-O_{eq} N-(4-azido-5 ^{125}iodo salicyloyl)-glycyl ryanodine (AzSGRy). In equilibrium binding affinity assays, non radiolabeled AzSGRy demonstrates an IC_{50} of 50nM. Using AzSGRy (0.5nM) as the radio-ligand in binding affinity assays, we found two distinct, sequential ryanodine binding isotherms residing within our normal 0 to 300nM ryanodine sigmoidal curve. The high affinity site demonstrates an IC_{50} of 0.5nM (plateau occurring between 3 and 6nM ryanodine), while the lower affinity site demonstrates an IC_{50} of 15nM. Scatchard analysis of direct binding of AzSGRy to junctional or heavy SR vesicles also revealed the presence of two classes of binding sites. Using AzSGRy, we demonstrate directly for the first time the presence of two distinct components in a typical ryanodine binding isotherm. Supported in part by the Showalter Trust Fund

Th-Pos350

CORRELATION OF RYANODINE'S BINDING WITH ITS INTRINSIC ACTIVITY ON THE SARCOPLASMIC RETICULUM CALCIUM-RELEASE CHANNEL (SR-CRC). ((S. Kwon, K.R. Bidasee, H.R. Besch, Jr., R.A. Humerickhouse and J.T. Emmick)) Dept. of Pharmacol. and Toxicol., Indiana Univ. Sch. of Med., Indianapolis, IN 46202. (Spon. by R. Haak)

Ryanodine, a complex alkaloid from the plant *Ryania speciosa* Vahl demonstrates a concentration-dependent, biphasic modulating action on the SR-CRC of striated muscle. In calcium flux experiments, this compound activates (opens) the SR-CRC at concentrations ranging from 10nM to 30μM (EC_{50} of 2.5μM) whereas higher concentrations deactivate (close) the channel (EC_{50} of 280μM). However, these concentrations are much higher than the concentration of ryanodine (300nM) required to saturate the receptor in equilibrium binding affinity assays (IC_{50} of 6.2 nM). As such, binding and functional studies have failed to establish unequivocally, a direct relationship between occupancy of ryanoid receptors and capacity to activate or deactivate the SR-CRC. We report here the first successful attempt to correlate directly, ryanodine binding with its activity on the SR-CRC. Using the novel photoactivatable derivative of ryanodine C_{10}-O_{eq} N-(4-azido salicyloyl) glycyl ryanodine (AzGlyRy) which demonstrates a similar biochemical and pharmacological profile to ryanodine, as a competitive, irreversible antagonist, we were able to relate the decrease in ryanodine receptor availability to [^{3}H] ryanodine binding in equilibrium binding affinity assays (as a function of the amount of ryanodine bound) with the calcium loss from the heavy SR vesicles (JSRV). Maximum calcium loss from JSRV is achieved at receptor occupancy of 6nM ryanodine (EC_{50} of 0.6nM). Interestingly, receptor occupancy up to 25nM ryanodine (equivalent to 75% decrease in [^{3}H] ryanodine binding) continues to maximally activate the SR-CRC. Concentrations of receptor occupancy greater than that occurring at 25nM ryanodine progressively deactivated the SR-CRC. Supported in part by the Showalter Trust Fund.

Th-Pos351

COMFA ANALYSIS OF RYANODINE BINDING TO VERTEBRATE SKELETAL MUSCLE RYANODINE RECEPTORS. ((W. Welch, K. Mitchell, L. Ruest, P. Deslongchamps, S. Ahmad, K. Gerzon, H.R. Besch, Jr., J. A. Airey, P. Verhey and J. Sutko)) Depts. Biochemistry and Pharmacology, U. Nevada, Reno, 89557; Dept. chimie, U. Sherbrooke, Quebec J1K 2R1; Dept. Pharmacology, Indiana U., Indianapolis, 46223.

The global conformational minima of a series of synthetic and natural ryanodine analogs were determined using molecular mechanics and dynamics. The relationship between structure and the experimentally determined binding to vertebrate ryanodine receptor was analyzed by comparative molecular field analysis (CoMFA) using an sp3 hybridized carbocation as the probe atom. The regions of dominant steric and electrostatic interactions were mapped. Data analysis indicates adding or removing an exocyclic functional group perturbs binding directly as well as indirectly by inducing conformational changes remote from the site of modification. This indirect effect can be larger than the direct effect. The CoMFA model has been found to predict the dissociation constants of novel ryanoids with excellent reliability even when large alterations in the location of the pyrrole group have been made.

Th-Pos352

DIRECT QUANTIFICATION OF CALCIUM BINDING TO THE PURIFIED RYANODINE RECEPTOR.
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The ryanodine receptor has been shown to be the calcium channel responsible for the release of calcium out of the sarcoplasmic reticulum (SR). Several modifiers of both channel gating and ryanodine binding have been previously described, such as ATP, Mg^{2+}, ruthenium red and Ca^{2+} itself. Recently different regions of the protein have been proposed as putative sites for calcium binding. However, at this time, Ca^{2+} binding to purified ryanodine receptor has never been directly quantified.

We have developed a technique allowing the measurement of ^{45}Ca binding to the ryanodine receptor. Ryanodine receptor, purified from rabbit skeletal muscle, was incubated in presence of different concentrations of ^{45}Ca and then filtrated through an Immobilon membrane. The measurement of Ca^{2+} binding to the ryanodine receptor as a function of Ca^{2+} concentration yields an affinity for Ca^{2+} of 20 μM. This value is in good agreement with the one obtained for the effect of Ca^{2+} on Ca^{2+}-release from SR vesicles or on channel gating. Thus this Ca^{2+} binding is likely to correspond to the high-affinity Ca^{2+} binding site responsible for the activation of the Ca^{2+} channel. This binding is completely abolished in presence of ruthenium red, but not affected by the presence of thapsigargine which has been shown to inhibit Ca^{2+} binding to the Ca^{2+}-ATPase. The effects of magnesium as well as ryanodine on the binding of Ca to the ryanodine receptor are investigated.

Supported by the Association Française contre les Myopathies (AFM).

Th-Pos353

PHYSIOLOGICAL DIFFERENCES BETWEEN THE α AND β RYANODINE RECEPTORS OF FISH SKELETAL MUSCLE. ((John O'Brien[1], Hector H. Valdivia[2] and Barbara A. Block[1])). [1]University of Chicago and [2]University of Maryland at Baltimore.

In mammalian striated muscle, ryanodine receptors (RYR) occur in two forms. A skeletal isoform is expressed in fast- and slow-twitch skeletal muscle and a cardiac isoform is expressed in heart. Despite extensive sequence homology between the two RYRs, skeletal receptors display a larger channel conductance and more readily inactivate when cytosolic calcium reaches millimolar levels. In fish, the majority of skeletal muscles express two RYR isoforms (α and β) together while certain muscles specialized for fast contraction express only α. The functional implications of this expression pattern are unknown but important given that models of the mechanism of EC coupling are based on results from fish, amphibians and mammals. Using [^{3}H] ryanodine binding as an indicator of channel activity, and reconstituting Ca^{2+} release channels into planar lipid bilayers, we have studied the Ca^{2+}-dependence of channel activity of the α and β isoforms from fish. In extraocular and swimbladder muscles, which express the α isoform alone, [^{3}H] ryanodine binding has a threshold for activation at 100 nM Ca^{2+} and peaks at 10 μM Ca^{2+}. 3 mM Ca^{2+} completely inactivates binding. This biphasic response results in a bell-shaped curve similar to that of rabbit skeletal muscle. Fish cardiac RYR displays a similar activation threshold but shows optimal [^{3}H] ryanodine binding at 100 μM Ca^{2+} and only 20% inactivation by 3 mM Ca^{2+}. This curve is similar to that of pig and bovine heart. In swimming muscle, which expresses both α and β isoforms, the Ca^{2+}-dependence of binding is a blend of cardiac and skeletal features, being optimal at [Ca^{2+}] similar to cardiac and inactivating 40% at 3 mM Ca^{2+}. In single channel recordings from swimming muscle SR, two different calcium channels are identified based on unitary channel conductance and Ca^{2+} sensitivity. A 520 pS channel displays maximal open probability when cytosolic Ca^{2+} is between 10 and 100 μM and is completely inactivated by 1 mM Ca^{2+} closely following ryanodine binding to the α isoform. In contrast, a 380 pS channel shows little inactivation at 1 mM Ca^{2+} similar to ryanodine binding to the cardiac RYR. We propose that the α and β RYR of fish muscle are functional counterparts of mammalian skeletal and cardiac RYR respectively. Supported by NIH AR40246 to BAB and AHA to HV.

Th-Pos354

DEACTIVATION OF SR CALCIUM-RELEASE CHANNEL REFLECTS CROSSLINKING AMONG MONOMERS. ((K.R. Bidasee, S. Kwon, H.R. Besch, Jr., R.A. Humerickhouse and J.T. Emmick)) Dept. of Pharmacol. and Toxicol., Indiana Univ. Sch. of Med., Indianapolis, IN 46202. (Spon. by G. Nicol)

The plant alkaloid ryanodine is a potent and specific modulator of the sarcoplasmic reticulum calcium-release channel (SR-CRC). In calcium flux experiments, this compound produces a biphasic response: at concentrations ranging from 10nM to 30μM, it activates (opens) the SR-CRC (EC_{50} of 2.5μM), whereas higher concentrations deactivate (close) the channel (EC_{50} of 280μM). To date, little is certain regarding whether the dual actions of ryanodine are due to the alkaloid binding to functionally distinct sites or to conformational changes occurring within the receptor subsequent to the binding of ryanodine. We provide here the first direct evidence that the deactivating effect of ryanodine results from polymerization of receptor monomers. Utilizing a novel, photo-activatable derivative of ryanodine (C_{10}-O_{eq} N-(4-azido salicyloyl) glycyl ryanodine, (AzGlyRy)), which has a similar biochemical and pharmacological profile to ryanodine (under equilibrium binding affinity assay conditions AzGlyRy demonstrates an IC_{50} of 40nM and under efflux conditions activates the SR-CRC at concentrations up to 1mM (EC_{50} of 60μM) and deactivates the channel at higher concentrations) we were able to covalently bind the ligand to the receptor thereby preserving "the state of the channel" and measure the associated loss in calcium from heavy sarcoplasmic reticulum vesicles (JSRV). In addition, SDS PAGE of the JSRV after treatment with AzGlyRy (0 to 700μM) revealed a progressive decrease in ryanodine receptor monomers (565 kDa) and low molecular weight proteins (<10 kDa) and the simultaneous appearance of two new protein bands, one at the interface of the stacking and resolving gels and the other at the top of the stacking gel. It is likely that these represent dimeric and tetrameric forms of the receptor.

Supported in part by the Showalter Trust Fund

Th-Pos355

ANION CHANNEL ACTIVATED BY INTRACELLULAR CA^{2+} IN RABBIT CARDIAC VENTRICULAR MYOCYTES.

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In cardiac myocytes, Ca^{2+} sensitive $I_{to}(I_{toCa})$ is known to be activated during rapid stimulations as the concentration of intracellular $Ca^{2+}([Ca]_i)$ increased. However the activation mechanism of I_{toCa} by $[Ca]_i$ is not well understood. We examined how $[Ca]_i$ affected the generation of I_{toCa} in rabbit ventricular myocytes by patch clamp method. When $[Ca]_i$ was changed between 1 nM and 1 μM Ca^{2+}, I_{toCa} could be elicited by the depolarizing pulses with $\geq$ 10 nM Ca^{2+}. The amplitudes of I_{toCa} were increased with rasing $[Ca]_i$ in a concentration dependent manner. Time- and voltage-dependent inactivations were faster at higher $[Ca]_i$ in all voltages. While the steady state activation and inactivation were independent on $[Ca]_i$, $V_{1/2}$ for activation and inactivation were +42 mV and -35 mV, respectively. I_{toCa} was completely inhibited by replacement of Cl^- with aspartate or application of anion transporter blocker(DIDS). While decreasing Cl^- concentration in the bath solution from 150 mM to 10 mM, the reversal potential shifted from 0 mV to +60 mV, indicating I_{toCa} carried by Cl^-. We have determined the anion permeability sequence of I_{toCa} as SCN^- (2.22), I^- (2.21) > Br^- (1.6) > Cl^- (1.0). From these results, it is concluded that I_{toCa} is a Ca^{2+}-activated anion channel, which is activated in pysiological ranges of $[Ca]_i$ and has time- and voltage-dependent properties, indicating another regulatory mechanism by $[Ca]_i$ in heart.

Th-Pos356

A Ca^{+2}-DEPENDENT TRANSIENT OUTWARD CURRENT IS PRESENT IN MYOCYTES OF THE EPICARDIAL BORDER ZONE FROM INFARCTED CANINE HEARTS.

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Studies have shown the presence of two types of transient outward currents in myocytes from normal hearts. One type is blocked by 4-AP and carried by K^+ ions (I_{to1}), while the other type is Ca_i-dependent (I_{to2}). Others have shown I_{to2} to be carried by Cl^- ions and blocked by caffeine, ryanodine (RYAN), or anion transport blockers. In this study we examined I_{to2} in epicardial myocytes from normal noninfarcted hearts (NZs) and those from epicardial border zone of the 5 day infarcted heart (IZs). We recorded I_{to2} using whole cell voltage clamp techniques under conditions to remove contaminating currents (5mM Ca_o,0 Na_{o+},0 K,3mM EGTA in pipette, 2mM 4-AP, 36-37°C). In NZs and IZs, we found I_{to2} was not completely abolished with 0.2mM diisothiocyanostilbene 2,2-disulphonic acid (DIDS) (70-80% block) but required the addition of 2 μM RYAN for complete block. The DIDS+RYAN-sensitive difference currents were small in 2 of 6 IZs, but showed dramatic changes in peak I-V relationships and amplitudes in 4 others; IZs (peak 904 to 1845pA), NZs (peak 614 to 1200pA,n=4). Decay of DIDS-sensitive currents during step depolarizations to positive test potentials showed little voltage dependence (τ=7-11ms) in NZs and IZs. When $I_{Ca,L}$ was reduced by prepulsing to positive potentials, the size of DIDS-sensitive difference outward tail currents elicited by a step to +80mV were also reduced. Amplitudes of these outward tails were larger in IZs (range 728-2122pA) than in NZs (range 733-938pA). Time constants of decay of outward tails were monoexponential: 5.9-7.4ms in NZs; 6.3-7.0ms in IZs. These findings suggest 1) I_{to2} exists in NZs and IZs in the absence of catecholamines and 2) I_{to2} is more prominent in most IZs. This could contribute to the altered time course of repolarization observed in IZs.

Th-Pos357

VOLTAGE-SENSITIVE CHLORIDE CHANNELS RECONSTITUTED FROM CARDIAC SARCOLEMMA IN PLANAR LIPID BILAYERS.

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We have identified and characterized novel voltage-sensitive Cl^- channels that are abundant in porcine ventricular sarcolemma. The conductance of the channels is 85 pS in symmetrical 200 mM KCl, and there is a prominent 42 pS substate. The main conductance shows simple saturation behavior with increasing KCl concentrations, with a K_m of 900 mM and maximum conductance of 488 pS. The channels are highly selective for Cl^- over K^+ and Na^+, with P_{Cl}/P_K=80 and P_{Cl}/P_{Na}=75. The channels are highly permeable to halide and polyatomic anions; using bi-ionic conditions, we find a permeability sequence of $SCN^- > NO_3^- \sim Br^- > Cl^- > F^- > HCOO^-$. The channels display a moderate voltage sensitivity at normal cellular potentials; open probability rises monotonically from 0.35 at -80 mV to ~1 at -20 to +80 mV. These data are fit well by a simple Boltzmann distribution indicating half-maximal activity at -60 mV, and a gating charge of ~1. The channel mean open times are approximately 10 ms at -80 mV and increase to ~100 ms at -20 mV (with 200 Hz filtering). The channels are not Ca^{2+}-dependent, and their gating and open probability are independent of internal and external Ca^{2+} concentrations ranging from 10 μM to 20 nM. The channels are partially blocked by 100 μM SITS or DIDS, and are completely (and reversibly) blocked by 200 μM Cd^{2+} or 1 mM Zn^{2+}. We plan to evaluate the open- and closed-time distributions, the sensitivity of open- and closed-times to the voltage, and the effect of cellular regulatory components.

Th-Pos358

IDENTIFICATION OF $CFTR_{cardiac}$ IN NON-HUMAN PRIMATE VENTRICULAR MYOCYTES.

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The cAMP-dependent Cl^- conductance in rabbit and guinea-pig ventricular myocytes has previously been shown to be due to cardiac expression of an alternatively spliced isoform of CFTR ($CFTR_{cardiac}$). The possible expression of $CFTR_{cardiac}$ in primate heart has not yet been investigated. We used both electrophysiological and molecular techniques to examine the expression of $CFTR_{cardiac}$ in non-human primate ventricular myocytes. Electrophysiological experiments employed the whole-cell variation of the patch-clamp technique in enzymatically dispersed simian ventricular myocytes. Under appropriate conditions to inhibit sodium, potassium, and calcium currents, application of forskolin (FSK, 1 μM) evoked an increase (330.1 $\pm$ 100.7 pA at +50 mV, n=6) in a time-independent background current which was readily reversible upon washout of FSK. The reversal potential of this FSK induced current was shifted from -28.5 $\pm$ 2.8 mV (predicted E_{Cl} -32 mV) in a normal (136mM) Cl^- external solution to -2.3 $\pm$ 4.5 mV (predicted E_{Cl}, 0mV) in a low (40 mM) Cl^- external solution (n=3). Molecular analysis employing the reverse transcription-polymerase chain reaction technique demonstrated expression of $CFTR_{cardiac}$ in both simian atrial and ventricular tissue. We amplified four distinct regions corresponding to transmembrane segments I-VI, NBD1, transmembrane segments VII-XII, and the R domain. Amplification of transmembrane segments I-VI indicated that, as in other species, simian heart expresses an alternatively spliced form of CFTR. (Supported by NIH HL30143).

Th-Pos359

DIVERSITY OF EXPRESSION OF CFTR Cl^- CHANNELS IN HEART.

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Published electrophysiological data indicates that there may be significant diversity in the expression of cAMP-dependent Cl^- channels in different areas of the heart and in different species. We previously determined that the cAMP-dependent Cl^- conductance in heart is due to cardiac expression of an alternatively spliced isoform of the epithelial CFTR Cl^- channel (Am. J. Physiol. 264:H2214, 1993). We used electrophysiological and molecular techniques as well as *in situ* hybridization to examine the expression of CFTR Cl^- channels in atrium and ventricle of guinea-pig, rabbit and dog hearts. cAMP elicited robust time-independent Cl^- currents in myocytes isolated from guinea pig and rabbit ventricle but not in guinea-pig and rabbit atrium or in dog atrium and ventricle. We used the polycrase chain reaction (PCR) to amplify three distinct regions corresponding to the cardiac CFTR gene product from each of these cardiac tissues to determine if the molecular distribution of CFTR matches the distribution of cAMP-dependent Cl^- channels. Amplication of regions corresponding to NBD1, transmembrane segments VII-XII, and the R domain showed a precise correlation to tissues which electrophysiologically exhibit cAMP-dependent Cl^- channels. Thus fragments corresponding to each of the three CFTR regions could be amplified from guinea-pig and rabbit ventricle, but not from guinea-pig and rabbit atrium or from dog atrium or ventricle. A ^{35}S riboprobe based upon the cDNA encoding a region of the rabbit cardiac CFTR Cl^- channel was hybridized *in situ* to rabbit ventricular endocardial and epicardial tissue and to rabbit right atrial tissue. Labelling by the CFTR probe could be detected in ventricular epicardial tissue but not in ventricular endocardial or atrial tissue. This distribution of CFTR in rabbit heart closely matches the electrophysiological distribution of cAMP-dependent Cl^- channels in rabbit heart (Takano and Noma, 1992). (Supported by HL-30143, AHA and AHA Nevada Affiliate).

Th-Pos360

"REVERSE AGONIST" EFFECT OF ATROPINE ON PKA-REGULATED Cl CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES.

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We investigated effects of atropine (Atr), a muscarinic antagonist, on the Cl current regulated by cAMP-dependent protein kinase (PKA) in cardiac myocytes. Whole-cell currents were recorded at ~36 °C in myocytes voltage-clamped and internally dialyzed with narrow-tipped pipettes (~5 MΩ). With 20 mM pipette [Cl] and 150 mM extracellular [Cl], bath application of isoproterenol (Iso; 10-30 nM) activated typical Cl conductance. The Cl conductance was increased to ~130% by Atr (1 μM) whereas it was reduced to ~10% by carbachol (CCh; 1 μM). In the absence of Iso, Atr (10 nM-1 μM) alone had no effect. When 1 μM Iso was used, Atr no longer enhanced the Cl conductance. The Atr stimulatory action was abolished by a 1-2 hr incubation of myocytes at 36°C with 5 μg/ml PTX. Thus, Atr itself can act as a "reverse agonist" to attenuate basal activity of inhibitory G proteins (G_i) by stabilizing inactive forms of muscarinic receptors. Adenosine (Ado 1 μM), known to stimulate G_i via A_1 receptors, also reduced the Cl conductance. Upon cessation of G_i stimulation by either Ado or CCh, a transient over-recovery of the Iso-activated conductance was observed. Since no rebound was seen when cells were vigorously dialyzed with 100 μM GTP or when 1 μM Iso was used, it might reflect reduced basal activity of G_i caused by local GTP depletion due to enhanced hydrolysis. Atr, or 8-sulfophenyltheophyline (50 μM; an A_1 antagonist), substantially potentiated the rebound associated with CCh or Ado, presumably by acting as reverse agonists and therefore enhancing the reduction of G_i activity. *(Supported by Grants-in-Aid for Scientific Research from the Japan Ministry of Education, Science and Culture)*

Th-Pos361

ANION PERMEABILITY SEQUENCE OF THE OPEN CARDIAC CFTR Cl CHANNEL. ((A.G. Dousmanis & D.C. Gadsby)) Laboratory of Cardiac/Membrane Physiology, The Rockefeller University, New York, NY 10021. (Spon. by T. Sakmar)

PKA-phosphorylated CFTR Cl channels continuously open and close in guinea pig ventricular myocytes dialyzed with ATP-containing solutions, but become "locked" open when pipette solutions also contain the non-hydrolyzable ATP analogue, AMP-PNP. We determined anion permeability sequences under both conditions to test for possible confounding effects of substituent anions on CFTR channel gating. In myocytes exposed to 125 mM extracellular Cl and dialyzed via wide-tipped perfused pipettes containing 125 mM Cl, steady-state whole-cell current-voltage (I-V) relationships were determined using 80-ms voltage pulses to potentials between -100 and +100 mV from the 0 mV holding potential, in the absence and in the presence of activated CFTR Cl conductance (by 2 μM forskolin or 1 μM isoproterenol). In both of those conditions, I-V relationships were obtained immediately before, during, and after brief (~30 s) equimolar substitutions of the test anions, NO_3, F, I, Br, and aspartate (Asp), for extracellular Cl. These brief exposures minimized compromise of the biionic assumption by entry of the test anion into the cell, as well as possible intracellular effects of the anions on background conductances and on regulation of CFTR channels. PKA-activated I-V relationships were obtained by appropriate subtraction and relative permeabilities, P_X/P_{Cl}, were then determined from the measured shifts of reversal potential, $\Delta E_{rev} = E_{rev}(X_o) - E_{rev}(Cl_o)$, using $\Delta E_{rev} = (RT/zF)\ln(P_X/P_{Cl})$. The sequence of relative permeabilities was (n=4-8) NO_3 (1.69±0.03, SEM)>Br (1.51±0.06)>I (1.29±0.11)> Cl (1.0)>F (0.20±0.04)>Asp (0.07±0.01), with 4-10 mM pipette ATP, and was (n=3-5) NO_3 (1.82±0.07)>I (1.32±0.05)>Cl (1.0)>F (0.31±0.05), with 2 mM ATP + 2 mM AMP-PNP in the pipette. The permeability sequence I>Cl contrasts with earlier reports for epithelial (Cl>I) and cardiac (Cl≥I) CFTR conductance. However, extracellular I diminished both outward current at positive potentials and Cl efflux at negative potentials, in agreement with previous work, independent of the presence or absence of channel gating. The latter observation suggests that I readily enters open CFTR channels but leaves them only slowly. Supported by NIH.

Th-Pos362

SULFONYLUREAS CAUSE OPEN CHANNEL BLOCKADE OF CFTR. ((C.J. Venglarik, A.D.G. DeRoos, A.K. Singh, B.D. Schultz, R.A. Frizzell and R.J. Bridges)) Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL, 35294.

Glibenclamide and tolbutamide reduce CFTR whole cell currents (Sheppard and Welsh, J. Gen. Physiol. 100:573, 1992). The aim of this study was to quantify and compare possible effects of these sulfonylureas on CFTR gating. Excised inside-out patches were obtained from CFTR-transfected mouse L cells and perfused with a NaCl bath (37°C) containing ATP (300 μM) and $MgCl_2$ (2.3 mM). Addition of either glibenclamide or tolbutamide reduced P_o and the resulting concentration-response relations were consistent with Michaelis-Menten type inhibition. The K_i was 54 μM for glibenclamide and 443 μM for tolbutamide. Current fluctuation analysis of multi-channel records revealed that addition of either sulfonylurea caused a new Lorentzian component to appear in the PDS. In both cases the corner frequency (f_c) of the Lorentzian increased as a linear function of blocker concentration, which is the result expected for open channel blockade (Lindemann and Van Driessche, Science 195:292, 1977). The plot of $2\pi f_c$ as a function of tolbutamide concentration provided estimates of k_{off} (1500 s^{-1}), k_{on} ($5.5 \cdot 10^6$ $M^{-1}s^{-1}$) and K_i (273 μM). The plot of $2\pi f_c$ as a function of glibenclamide concentration yielded estimates of $k_{off} + k_{close}$ (31 s^{-1}) and k_{on} ($2.0 \cdot 10^5$ $M^{-1}s^{-1}$). These results indicate that tolbutamide has a ≈25-fold greater k_{on} than glibenclamide but is a less potent blocker due to a >100-fold increase in k_{off}. Supported by NIH DK45970, DK42017, DK38581 & HL07553.

Th-Pos363

CPX ACTIVATES CHLORIDE EFFLUX FROM CELLS EXPRESSING MUTANT, BUT NOT WILD TYPE CFTR. **((O. Eidelman, C. Guay-Broder, L. Vergara and H.B. Pollard)) LCBG, NIDDK, NIH, Bethesda, MD 20892.**

In order to assess the potential of 8-cyclopentyl-1,3-dipropyl-xanthine (CPX) in overcoming defective chloride transport in CF cells, we studied its effects on the activation of chloride efflux from two CF cell lines expressing different CF mutations and 3T3 cells stably transfected with wild type (wt) or ΔF508 CFTR. CPX enhanced chloride efflux in CF cells bearing two different mutations, ΔF508 (CFPAC-1) and ΔF508/W1282X (IB3). In either case, correction of the CF defect by transfection with wt CFTR rendered the cells insensitive to CPX. The response to CPX was correlated to the presence of mutant CFTR by using 3T3 fibroblasts transfected with either wt or ΔF508 CFTR and CFPAC-1 cells transfected with wt CFTR. In all cell types expressing ΔF508, CPX was found to activate chloride efflux in a dose dependent manner. We also found that Cl^- efflux fron CFPAC cells had two components: one was immobile at 19°C while the other depended on K^+ gradient. We conclude that CPX can activate chloride efflux from cells which express ΔF508 CFTR but only if wt CFTR is absent, thus implying that CPX effects on normal cells or on cells which do not express CFTR are minimal.

Th-Pos364

NUCLEOTIDE BINDING OF CFTR AND ASSOCIATED PROTEINS. ((S. Bar-Noy, O. Eidelman and H.B. Pollard)) LCBG, NIDDK, NIH, Bethesda, MD 20892.

The function of CFTR, one of the ABC super family, is regulated by phosphorylation and by ATP which may interact with CFTR in the nucleotide binding fold domains. However, nucleotide effects on CFTR might also be due to action on other proteins which may be associated with CFTR *in vivo*. To further examine this hypothesis we have prepared immunoprecipitates of CFTR under non-denaturing conditions and used them in nucleotide binding assays. This approach has allowed us to ask whether CFTR indeed binds ATP and also if these nucleotide binding properties are shared by any of the proteins which are co-isolated with CFTR. The critical question is whether these proteins are truly complexed with CFTR or just adhere by non-specific means. Adequate measures were taken in order to test these possibilities. Labeling of immunoprecitates was by phosphorylation with [γ^{32}P]-ATP or by photoaffinity labeling with [α^{32}P]-8-azido-ATP. Competition with cold nucleotides was used to test for specific labeling. Several labeled bands were found to be common to all precipitates. We conclude that CFTR specifically binds ATP and that a limited number of co-immunoprecipitated proteins are also able to bind nucleotides.

Th-Pos365

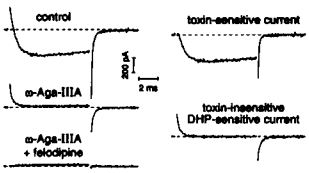
EPITHELIAL CFTR CHLORIDE CURRENT EXHIBITS OUTWARD RECTIFICATION DEPENDENT UPON INTRACELLULAR ANIONS. ((J.L. Overholt, A. Saulino, M.L. Drumm, and R.D. Harvey)) Dept. of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106.

The disease cystic fibrosis is caused by mutation of a gene which codes for a cAMP-dependent Cl^- channel, CFTR, found in epithelial cells. Current-voltage (I-V) relations attributed to CFTR are generally referred to as being linear to distinguish them from a separate, large conductance, outwardly rectifying Cl^- channel also found in epithelial cells. However, cardiac myocytes express an alternatively spliced isoform of CFTR, and we have previously shown that activation of this isoform produces whole-cell Cl^- currents that exhibit rectification dependent on the anions present in the intra, but not the extracellular solution (*J. Gen. Phys.* 102: 871-896, 1993). We used the whole-cell configuration of the patch clamp technique to investigate whether epithelial CFTR Cl^- currents behaved in a similar manner. Currents were activated by forskolin and IBMX in T84 epithelial cells and 293 cells transfected with wild-type CFTR. When intracellular Cl^- (Cl^-_i) was reduced by equimolar replacement with glutamate, with 150 mM extracellular Cl^- (Cl^-_o), I-V relations were outwardly rectifying. Increasing Cl^-_i from 40 to 80 and 150 mM (150 mM Cl^-_o) decreased rectification in a concentration dependent manner, such that I-V relations with symmetrical high (150 mM) Cl^- were nearly linear. Elimination or reversal of the Cl^- gradient by lowering Cl^-_o from 150 to 40 mM had no effect on the strength of rectification at a given Cl^-_i, indicating that rectification is not simply due to the Cl^- concentration gradient. Further, rectification was less when Cl^-_i was reduced by equiosmolar replacement with sucrose instead of glutamate. This demonstrates that physiological anions other than Cl^-_i can affect whole-cell CFTR currents in epithelial cells, and, that Cl^- currents in epithelial cells are qualitatively and quantitatively similar to those found previously in cardiac myocytes.

Th-Pos366

ω-Aga-IIIA ISOLATES L-TYPE Ca CHANNEL GATING CURRENTS.
((Eric A. Ertel, McHardy M. Smith, Mark D. Leibowitz, and Charles J. Cohen)) Merck Research Labs, R80N-31C, PO Box 2000, Rahway, NJ 07065.

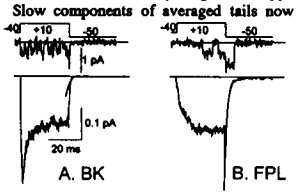
The peptide ω-agatoxin-IIIA (ω-Aga-IIIA) blocks ionic current through L-type Ca channels in guinea pig atrial cells without affecting the associated gating currents. Under conditions that isolate L-type Ca channel currents, ω-Aga-IIIA blocks all ionic current (I_{Ca}) and reveals opposite intramembrane charge movements at the beginning of a test pulse (Q_{on}) and after repolarization (Q_{off}). Q_{on} and Q_{off} are suppressed by 1 μM felodipine, saturate with increasing test potential, and are insensitive to Cd. The transient current associated with Q_{on} decays biexponentially and its slow time constant is similar to that for activation of I_{Ca}. The current associated with Q_{off} decays monoexponentially and more slowly than I_{Ca}. Similar charge movements are found in tracheal myocytes, which lack Na and T-type Ca channels. The kinetic and pharmacological properties of Q_{on} and Q_{off} indicate that they reflect L-type Ca channel gating currents. ω-Aga-IIIA has no effect on gating currents when I_{Ca} is eliminated by stepping to the reversal potential for Ca or by Cd block. In physiological [Ca], gating currents constitute a significant component of total current and they obscure the activation and deactivation of L-type Ca channels. Using ω-Aga-IIIA, we resolve the entire time course of L-type Ca channel ionic and gating currents. We also show that L- and T-type Ca channel currents can be accurately quantified by tail current analysis once gating currents are taken into account.



Th-Pos367

DISTINCTIVE ACTION OF FPL-64176 ENABLES DISSECTION OF N-TYPE AND L-TYPE Ca^{2+} CURRENT IN SYMPATHETIC NEURONS. *((B.A. Lewis, Jr. and D.T. Yue)), Johns Hopkins School of Medicine, Baltimore, MD 21205.*

Dihydropyridine agonists (DHP+) greatly slow tail-current deactivation of L-type Ca channels, but not other high-threshold Ca channels. In sympathetic neurons, this effect has proven an important tool for investigating selective regulation of macroscopic currents of L-type vs. N-type Ca channels (Plummer *et.al.* J Neurosci 11:2339), and for pursuing single-channel studies of N-type Ca channels requring definitive identification of patches with only N-type activity (Plummer *et.al.* Neuron 2:1453). While this strategy works when Ba^{2+} carries charge, we now find that DHP+ effects are greatly diminished in the physiological context when Ca^{2+} conducts current. With 120 mM Ca^{2+} as charge carrier for L-type Ca channels in frog sympathetic neurons, 1 μM Bay K 8644 (BK) only modestly prolongs test pulse openings, and hardly lengthens tail openings (A). The sum amplitude of slow exponentials fitted to ensemble-average tail currents is only 2% of steady-state test current, versus 50% when Ba^{2+} carries charge. Macroscopic tail currents from 10 to 15 msec after repolarization to -50 mV average to only 10% of peak test current with Ca^{2+}, but 40% with Ba^{2+}. Could the novel, non-DHP Ca channel agonist FPL 64176 (FPL) provide an alternative? Under conditions as in A with Ca^{2+}, 1 μM FPL markedly lengthens L-type openings, even during tail repolarization (B). Slow components of averaged tails now exceed preceding test current by 2-fold. Macroscopic tails from 10 to 15 msec after repolarization to -50 mV average to 70% of peak test currents, regardless of charge carrier. Importantly, the slow FPL-induced tails are insensitive to 3 μM ω-Cgtx, arguing for selective modulation of L-type activity. FPL can thereby dissect L-type from N-type activity, regardless of charge carrier.



Th-Pos368

P-TYPE CALCIUM CHANNELS IN BOVINE ADRENAL CHROMAFFIN CELLS: DOWN REGULATION BY LAMBERT-EATON SYNDROME ANTIBODIES.
((Sung H. Kim, Taick S. Nam and Yong I. Kim)) *Departments of Biomedical Engineering and Neurology, University of Virginia School of Medicine, Charlottesville, VA 22908.* (Spon. by J. Pancrazio)

We have previously reported that voltage-dependent Ca^{2+} channels in bovine adrenal chromaffin cells are sensitive to ω-agatoxin IVA (ω-AgTx), a synthetic peptide toxin specific to P-type Ca^{2+} channels (Kim & Kim, *Mol. Biol. Cell* 4 [suppl]: 428a, 1993). The toxin inhibits the depolarization-induced increases in membrane capacitance, indicating that the P-type channels in these cells also participate in exocytosis. To determine whether the Lambert-Eaton syndrome (LES) antibodies specifically react with P-type Ca^{2+} channels, we examined the efficacy of ω-AgTx in blocking I_{Ca} in cells treated with control or LES IgG. In normal untreated cells, application of ω-AgTx (1, 5, 10, 20, 40 and 100 nM) resulted in a dose-dependent inhibition of I_{Ca} with a K_d of 6.5 nM. Upon incubation with IgG's from 3 LES patients (2-3 mg/ml IgG for 1 day), I_{Ca} was reduced by 49 ± 4% (mean ± SEM, P<0.001, n=42 cells). Adding 100 nM ω-AgTx to the control cells caused I_{Ca} to decline by 45 ± 3% (P<0.001, n=31), similar to that found in normal untreated cells (43%, n=65). However, the toxin produced virtually no reduction (-3 ± 0.2%, n=42) in the cells pretreated with LES IgG; this ineffectiveness (P<0.001) presumably resulted from the antibody-mediated destruction of P-type Ca^{2+} channels. These results are consistent with the previous finding that LES IgG similarly down-regulates P-type Ca^{2+} channels in small-cell lung cancer cells (Viglione & Kim, *Soc. Neurosci. Abstr.* 19: 703, 1993) and that the presynaptic Ca^{2+} channels at the mammalian neuromuscular junction, the pathogenic target of LES IgG, are ω-AgTx-sensitive P-type channels (Kim *et al., Mol. Biol. Cell* 4 [suppl]: 428a, 1993) (Supported by NIH grant NS18607 and the Muscular Dystrophy Association).

Th-Pos369

P-TYPE CALCIUM CHANNELS IN HUMAN SMALL-CELL LUNG CANCER CELLS: DOWN REGULATION BY LAMBERT-EATON SYNDROME ANTIBODIES.
((M.P. Viglione, T.J. O'Shaughnessy and Yong I. Kim)) *Departments of Biomedical Engineering, Neuroscience & Neurology, University of Virginia School of Medicine, Charlottesville, VA 22908.* (Spon. by C. Huang)

Given the high incidence of small-cell lung cancer (SCLC), the Lambert-Eaton syndrome (LES) is classified as a paraneoplastic disease of the neuromuscular junction (NMJ), characterized by a deficient release of ACh. The pathogenic target of the LES antibodies are the prejunctional calcium channels, which have been found to be P-type at the mouse NMJ (Uchitel *et al., Proc. Natl. Acad. Sci* 89: 3330, 1992 and Kim *et al., Mol. Biol. Cell* 4 [suppl]: 428a, 1993). Although an exact source of antigenic stimulus has not been identified, SCLC cells do express voltage-gated Ca^{2+} channels and therefore antigenic stimulation by SCLC cells is an attractive model for LES. To determine whether LES IgG specifically down-regulates P-type Ca^{2+} channels, we examined the efficacy of the toxin in inhibiting I_{Ca} after treatment with control or LES serum/IgG (1-4 mg/ml IgG from 3 LES patients for 1-2 days). LES cells exhibited a 30% (n=54 cells) reduction in I_{Ca} compared to the control. ω-agatoxin IVA (ω-AgTx, 100 nM), a toxin specific to P-type Ca^{2+} channels, inhibited I_{Ca} by 39% (n=54) in SCLC cells (NCI-H146) incubated with control IgG and 55% (n=22) in small-cell carcinoma cells of the adrenal gland (NCI-H510A). In the cells treated with LES antibodies, however, the toxin produced only a 12% (n=54) decrement of I_{Ca}. These results indicate that the LES IgG down-regulates P-type Ca^{2+} channels. In view of the finding that ω-AgTx-sensitive, P-type channels are functional at the mammalian NMJ, we suggest that P-type Ca^{2+} channels expressed by SCLC cells are a source of antigenic stimulation for the production of pathologic antibodies in paraneoplastic LES (Supported by NIH grant NS18607 and the Muscular Dystrophy Association).

Th-Pos370

CHARACTERIZATION OF CALCIUM CURRENTS IN A HYBRID MOTONEURONAL CELL LINE.
((D.R. Mosier, P. Baldelli, O. Delbono, R.G. Smith, S.H. Appel, and E. Stefani)) Depts. of Neurology and Physiology & Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030. (Spon. by J. Dani)

To create a cell line expressing motoneuron properties relevant to the pathogenesis of the neurodegenerative disorder, amyotrophic lateral sclerosis (ALS), we performed somatic cell fusion of the N18Tg2 neuroblastoma cell line with embryonic rat ventral spinal neurons. This cell line (VSC4.1), when differentiated in dibutyryl-cAMP, expresses a high voltage-activated calcium current which is highly resistant to dihydropyridines and ω-conotoxin GVIA, but is blocked by 3-10 μM FTX, a polyamine toxin derived from venom of the funnel-web spider, *Agelenopsis aperta*. These pharmacological properties are similar to those of a calcium current described in motoneuron terminals (Uchitel et al., *P.N.A.S.* 89:3330, 1992). We have characterized these currents in VSC4.1 cells electrophysiologically in external solutions containing 40 mM Ba^{2+}, using the whole-cell patch technique. Ba^{2+} currents evoked by voltage steps from holding potentials of -70 mV or -50 mV typically activated near potentials of -40mV, with peak current amplitude observed between -10 and 0 mV. Half-maximal steady-state inactivation occurred near potentials of -30 mV, and paired-pulse inactivation was voltage-dependent with no obvious current dependence. Inactivation parameters were similar when Ca^{2+} instead of Ba^{2+} was used as the charge carrier. Peak currents evoked by voltage steps corresponded with peak tail current amplitudes, consistent with a relatively homogeneous current population in VSC4.1 cells. Of particular interest, Ba^{2+} currents in differentiated VSC4.1 cells are selectively increased following application of immunoglobulins purified from sera of ALS patients, suggesting a possible mechanism for initiation of cytotoxicity by ALS immunoglobulins which has been observed in this cell line (R.G. Smith et al., *P.N.A.S.*, submitted).

Th-Pos371

INHIBITION OF Ca^{2+}-CURRENT BY THE LIZARD VENOM TOXIN HELOTERMINE
((M. Nobile, L.D. Possani*, L. Spadavecchia and G. Prestipino)) Istituto di Cibernetica e Biofisica, CNR, Genova, ITALY. *Instituto de Biotecnologia/UNAM, Cuernavaca, MEXICO. (Spon. by M.Grattarola)

Helotermine (HLTx), a 25.5 kDa peptide toxin isolated from the Mexican beaded lizard *Heloderma horridum horridum* venom, was found to be a potent inhibitor of Ca^{2+}-channels in cerebellar granule cells of newborn rats. Macroscopic currents, carried by 10 mM Ba^{2+} were measured in the whole-cell configuration. The toxin at the saturating dose of 1.2 μM reversibly produced ≈ 50% block of voltage-dependent Ca^{2+}-current by a fast mechanism of action. Inhibition was concentration-dependent (with a half-effective dose of 170 nM) and voltage-dependent. The toxin did not affect the activation and inactivation kinetics of Ca^{2+}-currents. In the same preparation, block of Ca^{2+}-current by the P-type Ca^{2+}-channels blocker ω-Agatoxin IVA (ω-Aga-IVA) was also incomplete even at saturating doses (mean ≈ 50% at 200 nM). Instead, ω-Conotoxin GVIA (ω-Cgtx) at 5μM depressed ≈ 15% of the current and the dihydropyridine antagonist nifedipine block was ≈ 30% at 10 μM. HLTx inhibited the current resistant to ω-Aga-IVA with the same percentage but had smaller effect on the residual ω-Cgtx or nifepidine-resistant current. Our data suggest that the toxin selectively targets populations of Ca^{2+}-channels distinct from P-type channels.

Th-Pos372

CALCIUM CHANNELS FROM BOAR SPERM PLASMA MEMBRANES WITH L-TYPE CHARACTERISTICS: SENSITIVITY TO BAY-K ENANTIOMERS.
(Seema Tiwari-Woodruff, R. N. Peterson and T. Cox),)Department of Physiology, School of Medicine, Southern Illinois University, Carbondale, Il-62901.

The acrosome reaction, an important step for fertilization, is promoted by sperm-receptor and egg zona-ligand binding resulting in elevations of $Ca^{2+}{}_i$ and pH_i. We have been studying boar sperm plasma membrane calcium channels by incorporating them in artificial lipid bilayers. Bilayers were formed in either symmetrical cis-trans solution containing 50 mM NaCl, 10 mM HEPES, pH 7.4 with 100 mM $BaCl_2$ on the cis side as current carrying cation, or with 150 mM(cis) and 10 mM(trans) $BaCl_2$ gradient. Typically 50 mg of membrane vesicles were added to the cis side. Ba^{2+}-selective channels were easily identified at zero holding potentials as upward deflections (1-3 pA). Channel conductance was 15-18 pS in 100 Ba and 16-20 pS in 150 Ba. Dihydropyridine calcium channel agonists (±)-BAY-K 8644 (1-2 μM)c/t and S(-)-BAY-K 8644 (1 μM)c/t increased % open time and NPo by nearly 50 % at positive (+10 to +60 mV) holding potentials. DHP antagonists nitrendipine (1-10 μM) and R(+)-BAY-K (1-5 μM) decreased % open time and NPo by 25-30 %. The current voltage relationship was not modified by the DHPs. Pretreatment of channel with S(-)-BAY-K (1 μM) required higher concentrations of nitrendipine (10-100 μM) and R(+)-BAY-K (5 μM) to reduce % open time and NPo. Whereas pretreatment of antagonists at low concentrations did not remove the agonistic effects of S(-)BAY-K. We conclude that we have identified barium conducting channels in boar plasma membranes with L-type characteristics. These channels may be involved in mediating the calcium influx that immediately precedes the acrosome reaction.

Th-Pos373

MODULATION OF *XENOPUS* OOCYTES ENDOGENOUS AND EXPRESSED CALCIUM CHANNELS BY THE BETA SUBUNIT.
((A. Neely, R. Olcese, X. Wei, L. Birnbaumer, E. Stefani)). Dep. of Molec. Physiol. & Biophys. Baylor Col. Med. Houston, TX 77030.

Ca^{2+} channels are heteromultimeric proteins in which the largest component (α_1) forms the ionic channel. Coexpression of additional subunits, such as β, results in an up-regulation of expressed Ba^{2+} currents (I_{Ba}). In *Xenopus* oocytes, injection of mRNA encoding for the β subunit increase endogenous I_{Ba}. Both control and β induced currents were preferentially found in the animal pole of the oocytes. They were insensitive to dihydropyridine (DHP) blockers and had distinct kinetic properties. Native and β_4 induced I_{Ba} inactivated with a time constant of about 300 ms while currents induced by β_2 show slow and incomplete inactivation (≤70% after 6 sec). Activation and deactivation kinetics for β_2 induced currents were single exponential with time constant of less than 1 msec. In contrast, I_{Ba} currents from oocytes coexpressing α_{1C} and β_2 subunits were larger in the vegetal pole. They activated following a double exponential time course. The fast component was slower than for I_{Ba} induced by β_2 alone and contributed to half the current at 0 mV, independent of the expression level. From DHP block and kinetic properties, when α_{1C} message is present it appears that all the current expressed arise from an homogeneous population of $\alpha_{1C}\beta_2$ channels. In addition, we show that oocytes expressing β_2 currents can be transformed to $\alpha_{1C}\beta_2$ currents by post-injection α_{1C} mRNA.
(Supported by an NRSA fellowship to A. N. and grants HL37044 to L. B. and AR38970 to E. S. from NIH).

Th-Pos374

T-TYPE AND N-TYPE CALCIUM CHANNELS OF *XENOPUS* OOCYTES: EVIDENCE FOR SPECIFIC INTERACTIONS WITH BETA SUBUNITS.
((A.E. Lacerda, E. Perez-Reyes, X. Wei, A. Castellano, L. Birnbaumer and A.M. Brown)) Depts. of Molecular Physiology & Biophysics and Cell Biology and the Division of Neuroscience, Baylor College of Medicine, Houston, TX 77030

Expression of exogenous Ca^{2+} channel α_1-subunits in *Xenopus laevis* oocytes often requires the coexpression of accessory subunits either to produce detectable currents or to augment small expressed currents. Exogenous α_1-subunit single channel currents display complex kinetic and conductance properties. *Xenopus* oocytes possess endogenous Ca^{2+} channels. Although endogenous currents are small, expression of mammalian Ca^{2+} channel β subunits can increase their magnitude. We used the amplifying effects of β subunits in order to identify endogenous oocyte Ca^{2+} channels. Expression of rat brain β_4 increased macroscopic endogenous current magnitude with a small effect on kinetics. In contrast, expression of rat brain/cardiac β_2 produced a much larger increase in current magnitude and dramatically slowed current decay. Low concentrations of ω-conotoxin GVIA irreversibly blocked currents in both uninjected and β_2-injected oocytes. Single channel recordings revealed both T- and N-type calcium channels with conductances of 9 and 18 pS, respectively, in uninjected oocytes and in oocytes expressing either β subunit. Expression of either β subunit slowed average current decay of T-type single channels. N-type average current decay was slowed by expression of β_2 while β_4 had little effect. Supported by NIH grants HL-37044 and HL-046702.

Th-Pos375

DOCOSAHEXAENOIC ACID MODULATES NITRENDIPINE EFFECTS ON CALICUM CHANNELS AND CONTRACTION IN ADULT RAT CARDIAC MYOCYTES.
((K. Bogdanov, S. Pepe, A. Talo, H. A. Spurgeon, A. Leaf, H. Hallaq, and E. G. Lakatta)) Gerontology Research Ctr, NIA, Baltimore, MD 21224 and Mass. Gen. Hospital, Boston, MA 02114 (Spon. by P.H. Backx)

Dietary fish oil incorporates docosahexaenoic acid (DHA) into cell membranes. The effect of DHA on dihydropyridine interaction with L-type Ca^{2+} channel current (I_{Ca}) and twitch contraction (TC) in isolated myocytes (23°C) was measured. Nitrenpidine (N) (10 nM) reduced peak I_{Ca}, measured by whole cell clamp from -40 to -5 mV, and reduced TC amplitude, measured by photodiode during electrical stimulation (0.5Hz) by about 50%. DHA (5μM) abolished these N effects (table). DHA had no effect. Arachidonic acid (AA) did not block the N effects and had no effects alone (not shown).

	n	C,nA/μm	N, % C	DHA+N,%C	DHA, %C
I_{Ca}	7	0.7±0.2	21±7*	76±17#	86±11
TC	5	101±7	56±6*	82±9+	96±3

*p< 0.001 vs control (C); +p< 0.05, #p< 10^{-4} vs N.

That DHA abolishes the N effect but has no effect alone suggests that it binds to Ca^{2+} channels near N binding sites and modulates Ca^{2+} channel flux. This effect may be involved in the cardioprotective effect of fish oil diets *in vivo*.

Th-Pos376

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF HUMAN TYPE E CALCIUM CHANNEL.
((T. Schneider, X. Wei, N. Qin, R. Olcese, A. Neely, J. Costantin, P. Palade, E. Perez-Reyes, R.G. Smith, S.H. Appel, E. Stefani, L. Birnbaumer)). Dep. of Molec. Physiol. & Biophys. Baylor Col. Med. Houston, TX 77030.

L-type (DHP-sensitive) and non-L-type Ca^{2+}channels form two branches in an evolutionary tree and for each subfamiliy there are at least three genes known. Typical antagonists of non-L-type Ca^{2+}channels like ω-Aga-IVA, ω-CgTX MVIIC, ω-CgTX GVIA, and Ni^{2+} discriminate between a P-, Q-, R-, N-, and T-type Ca^{2+} currents. These antagonists are used to characterize cloned non-L-type α_1 subunits like α_{1A} (BI, CaCh-4), α_{1B} (BIII, CaCh-5), α_{1E} (BII, CaCh-6).

A 1 kb cDNA probe for non-L-type α_1-subunits was isolated from a mouse/rat hybrid cell line using PCR. It was homologous to rat α_{1B} subunit between domains IIIS5 and IVS5. Screening a human fetal brain cDNA library we isolated cDNAs of the human type α_{1A}, α_{1B}, and α_{1E}. The full length sequence of the huma α_{1E} subunit has an open reading frame of 6933 bp (M, 261 kDa). It has highest homology to the rabbit α_{1E} subunit (BII-1, 93%) showing 2 insertions: in the linker between domain II and III (7aa) and in the carboxyterminal region (43aa).

The cRNA encoding the α_{1E} subunit, when injected in *Xenopus* oocytes, gives rise to large inward current (≈70 μA/μF) using either 10 mM Ba^{2+} or Ca^{2+} and a single channel conductance of 19 pS in 79 mM Ba^{2+}. Currents appeared at -40 mV and reached a maximum at +10 mV. They inactivated by 50% with 1 or 10 sec prepulses to -30 mV and did not show Ca^{2+} dependent inactivation. (Gating current and modulation by β subunit are discussed by Olcese et al., this meeting). The currents were insensitive to 0.5 μM BAY K 8644, 100 nM ω-Aga IVA, 1 μM ω-CgTX GVIA, but sensitive to amiloride (1 mM), octanol (300 μM) and Ni^{2+} (IC_{50}≤50 μM). This pharmacology and the voltage dependence of activation and inactivation identify this channel with a 'mid-voltage activated' T-like Ca^{2+}current.

Th-Pos377

CALCIUM CURRENT DEVELOPMENT IN THE EMBRYONIC CHICK HEART.
((T.L. Creazzo and J. Burch)) Cellular Biology & Anatomy, Medical College of Georgia, Augusta, GA 30912-2000.

We have previously shown that both T- and L-type Ca currents are present in embryonic chick ventricular myocytes at embryonic age day 11 (Aiba & Creazzo, 1992, AJP, 262: H1182). In the present study, Ca currents were examined from embryonic days 4-17 using the perforated patch clamp (nystatin) with 1.8 mM Ca^{2+} in the extracellular solution. The average cell capacitance was the same for all cells used in this study regardless of age (5.7±0.2 pF; n=40). Between days 5 and 7 of incubation there is both a marked decrease in the total Ca current (8.1±1.9 and 2.2±0.7 pA/pF, respectively; test pulse = 0 mV) and a negative shift in the voltage dependence of activation and inactivation for $i_{Ca,T}$. Separation of $i_{Ca,T}$ and $i_{Ca,L}$ is not possible at day 5 as it is at later ages because voltage dependent activation and inactivation are similar for both currents. However, about half of the Ca current is blocked by 5 μM nifedipine while the remaining current is blocked by 200 μM Ni^+ indicating that both $i_{Ca,T}$ and $i_{Ca,L}$ are present at day 5. Interestingly, the chick heart is undergoing aortico-pulmonary septation between days 5 and 7 suggesting that hemodynamic changes may be occurring during this period. The peak magnitude of $i_{Ca,L}$ remains relatively constant from days 7-17 (about 2.5 pA/pF at +10 mV). However between days 11 and 17, $i_{Ca,T}$ decreased by greater than 3-fold (2.0±0.8 and 0.6±0.1 pA/pF, respectively at -30 mV). Postganglionic sympathetic innervation occurs coincidentally over this later period of development. The results suggest the possibility that Ca current development is influenced by events related to both structure and innervation of the heart. Supported by NIH HL36059 and HL39039 and a grant from the American Heart Association/Georgia affiliate.

Th-Pos378

A VOLTAGE-SENSITIVE CALCIUM CHANNEL α_1 SUBUNIT IN *DROSOPHILA MELANOGASTER* SHOWS ALTERNATIVE SPLICING AND CAUSES EMBRYONIC LETHALITY WHEN MUTATED

((L.M. Hall, D. Ren, D.F. Eberl, G. Feng, F. Hannan and W. Zheng)) Dept. of Biochemical Pharmacology, SUNY, Buffalo, NY 14260.

PCR based cloning has identified a calcium channel α_1 subunit cDNA from *Drosophila melanogaster* which shows highest sequence similarity to the rat brain type D α_1 subunit. The message for this *Drosophila* subunit is expressed throughout the embryonic nervous system and is first detected on Northern blots in 9-12 hour embryos. Genetic analysis of a point mutation has shown that this subunit plays a vital role in the organism since a premature stop codon in the cytoplasmic loop immediately following transmembrane segment IVS4 causes embryonic lethality. This embryonic lethality has been rescued following P-element mediated transformation with a ~40 kb cosmid genomic clone which contains the entire open reading frame for the subunit plus all the 5' upstream regulatory sequence required for normal expression of this gene. This upstream region can now be used to test the functional significance of alternatively spliced versions of this subunit. As a first step in this direction we have defined the intron/exon map of this gene and have identified alternative splice variants in two regions: in the cytoplasmic loop between IIS6 and IIIS1 and in a region in the vicinity of IIIS5. In both regions the multiple splice forms are expressed in a tissue specific fashion. Some of the splice variants in the IIS6/IIIS1 loop have potentially different phosphorylation patterns and thus might result in functionally distinct calcium channel subtypes.

Th-Pos379

GATING OF CALCIUM CHANNELS IN HUMAN MYOTUBES.

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Using the whole cell patch clamp technique we measured voltage-dependent Ca-inward currents in human myotubes derived from satellite cells. Biopsy material was processed for cell culture using the method of Brinkmeier et al, BBA 1145, 8-14 (1993). The experimental solutions equalled those used by Rivet et al, Cell Calcium 11, 507-514, 1990. It has been shown previously, that a conditioning depolarization could accelerate (frog fibres) or potentiate (rat myoballs) the L-type calcium current activation. Therefore, we carried out pulse protocols which would promote this effect. We applied strongly depolarizing prepulses of variable amplitude and length followed by a fixed test pulse which was separated from the prepulse by intervals of variable length and potential. Both the kinetics and the degree of activation of the slow current were not affected by the prepulse indicating a variability in the gating mechanisms of the L-current in different skeletal muscle preparations. We also investigated the question whether the gating of the channel is blocked by a dihydropyridine calcium antagonist. We blocked the current partially by nifedipine; flash photolysis of the drug several ms before a depolarizing step increased the subsequently activated current; during the depolarization the light flash led to a more rapid increase of the amplitude which shows that a voltage-dependent activation of the channel takes place despite the block by nifedipine.

Th-Pos380

COMPLEX BINDING KINETICS OF Ca^{2+} ANTAGONISTS TO L-TYPE Ca^{2+} CHANNELS: QUANTITATIVE ANALYSIS USING A MULTIPOINT ATTACHMENT MODEL OF DRUG BINDING.

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We have studied the dissociation of Ca^{2+}-antagonists from the dihydropyridine (DHP) and benzothiazepine (BTZ) binding domain from L-type Ca^{2+}-channels in skeletal muscle. The domains were specifically labeled with a fluorescent DHP (DMBODIPY-DHP) and a radiolabeled BTZ ((+)-cis-diltiazem, DIL), respectively. In accordance with earlier studies saturation analysis revealed the existence of a single high affinity binding site on the channel for both compounds.
Dissociation was induced either by 80-fold dilution of the incubation mixtures or addition of an unlabeled competitive inhibitor. With both ligands complex dissociation kinetics were observed incompatible with a simple bimolecular binding interaction. DMBODIPY-DHP dissociated in a biphasic manner. The dissociation rate constant (k-1) was only slightly dependent on the concentration of the unlabeled competitor ((+)-isradipine). Dilution-induced dissociation was faster than ligand-induced dissociation. In contrast, (+)-cis-DIL dissocated in a monophasic manner. The k-1 was accelerated about 10-fold when the concentration of unlabeled competitor ((+)-cis-DIL) was raised from 1 to 50 µM ("ligand-induced accelerated dissociation"). Dilution-induced dissociation was slower than ligand-induced dissociation. We quantitatively analyzed our binding data in terms of different binding schemes. The simplest binding schemes that fit our data and do not require complex structural assumptions, are based on the thermodynamics of non-covalent binding, where high affinity results from the cooperative interaction of a ligand molecule with multiple attachment points within its binding domain (Prinz H. and Striessnig J., J. Biol. Chem. 268, 18580, 1993). According to our model, ligand-induced accelerated dissociation is expected if at least two ligand molecules have simultaneous access to the same array of attachment points (DIL binding), but is prevented if the domain allows binding of only one molecule at a time (DHP binding). Multipoint attachment also accounts for a conformational rearrangement of the DHP-channel-complex, which is the simplest explanation for the observed biphasic dissociation kinetics of DMBODIPY-DHP.
This concept of multipoint-attachment can be successfully used to develop simple binding schemes for quantitative data analysis. We will demonstrate, that for many calcium antagonist binding data this approach provides structurally more plausible explanations than e.g. the assumption of allosteric interaction mechanisms.
This work was supported by grants from the Fonds zur Förderung der wissenschaftlichen Forschung, Austria (S6601, S6602) and the Dr. Legerlotz Foundation.

Th-Pos381

EXPRESSION OF A NOVEL CA^{2+} INFLUX PATHWAY IN Sf9 INSECT CELLS FOLLOWING INFECTION WITH RECOMBINANT BACULOVIRUS CONTAINING cDNA FOR THE TRANSIENT RECEPTOR POTENTIAL-LIKE (*TRPL*) PROTEIN OF *DROSOPHILA*.

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Activation of phospholipase C, elevation of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and stimulation of Ca^{2+} influx have been implicated in *Drosophila* phototransduction. Electrophysiological studies suggest that *trp* and *trpl* proteins may be important for the light-activated Ca^{2+} current found in *Drosophila* photoreceptor cells. Although these proteins exhibit homologies to voltage-gated Ca^{2+} and Na^+ channels, their actual function in insect cells and their relation to proteins involved in mammalian cell Ca^{2+} signaling remains unknown. In the present study, Ca^{2+} signaling was examined in fura-2-loaded Sf9 insect cells infected with baculovirus containing cDNA for the M5 muscarinic receptor alone (M5 cells) or in cells co-infected with both M5 and *trpl*-containing baculoviruses (M5-*trpl* cells). Addition of carbachol (Cch; 100 µM) to M5 cells increased $[Ca^{2+}]_i$ (mean ± SD; n=17) from 101 ± 20 nM to 762 ± 178 nM which declined to a sustained elevated level of 384 ± 102 nM after 3 min. The sustained component was eliminated by removal of extracellular Ca^{2+} or by addition of La^{3+} (10 µM). In M5-*trpl* cells, basal $[Ca^{2+}]_i$ was elevated to 217 ± 24 nM. Addition of Cch to M5-*trpl* cells produced a large increase in $[Ca^{2+}]_i$ to a sustained value of 677 ± 143 nM. This change in $[Ca^{2+}]_i$ was significantly attenuated in the absence of extracellular Ca^{2+}, but relatively insensitive to La^{3+}. To evaluate the contribution of Ca^{2+} influx to the overall profile observed, Ba^{2+}, a Ca^{2+} surrogate that is not a substrate for the Ca^{2+} pump, was used. Cch-stimulated Ba^{2+} influx was increased in M5-*trpl* cells relative to M5 cells. In contrast to M5 cells, a substantial component of Ba^{2+} influx in M5-*trpl* cells was insensitive to La^{3+}. These results suggest that expression of *trpl* is associated with appearance of a La^{3+}-insensitive Ca^{2+} entry pathway that may reflect either a *trpl*-dependent alteration of an endogenous Sf9 cell Ca^{2+} channel or formation of a novel Ca^{2+} influx pathway by the *trpl* protein. (Supported by NIH grants HL44119 and HL45198.)

Th-Pos382

A High-Conductance Voltage-dependent Multistate Ca^{2+} Channel Found in Sea Urchin and Mouse Spermatozoa.

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Key events in fertilization like the sperm acrosome reaction and egg activation involve ion fluxes through poorly understood channel-mediated mechanisms. The high sensibility of planar bilayers to detect single ion channels can be exploited to study gamete interaction and ion channel regulation. Previously we reported the characterization of a high conductance Ca^{2+}- selective, voltage-dependent, multi-state channel from isolated *S. purpuratus* sea urchin sperm plasma membranes fused to planar bilayers (Liévano *et al.*, 1990, *J. Gen. Physiol.* 95:273). We show here that functional ion channels can be transferred to planar lipid bilayers directly using spermatozoa from two sea urchins, *S. purpuratus* and *L. pictus*, and from mouse spermatozoa. Our studies show that spermatozoa from these species posses a conspicuous Ca^{2+}-selective, high conductance, multi-state, voltage-dependent channel. In the three species the channel displays a similar voltage dependence and equal $P_{Ba^{2+}}/P_{K^+}$ (~ 4). The presence of this atypical Ca^{2+} channel in such diverse species suggests it may play an important role in the physiology of these specialized cells.

Acknowledgments: Supported by CONACyT, DGAPA-UNAM. A. D. is an international scholar of the Howard Hugues Med. Inst.

Th-Pos383

THE ATP/ADP-INDUCED CALCIUM CHANNELS IN THE CHANG HUMAN LIVER CELL LINE.

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The ATP, ADP or γ-S-ATP-induced changes in cytoplasmic free calcium were studied using fura-2 fluorescence measurements. Chang cells (ATCC #CCL13) were grown on glass coverslips. Cells were loaded with 1.5 µM fura 2-AM for 25 min at 37° C, and experimental measurements were made by dual-wavelength fluorimetry. Dose-response experiments indicated that free cytoplasmic calcium reached a maximal (saturated) concentration at 1.0 µM ATP or ADP. There were no remarkable differences in time-dependence of the stimulus-induced calcium transitions. Using calcium-EGTA buffers, it was determined that a transient increase, which was followed by a decrease, in free cytoplasmic calcium was produced by calcium liberation from intracellular calcium stores and the activation of a calcium-pumping ATPase. All stimuli used were able to induce calcium conductance in the cell membrane. Time-dependence of the calcium conductance was different in calcium-free and 2mM calcium-containing buffers. The ATP/ADP-induced conductivity was not sensitive to classic calcium channel blockers; diltiazem, verapamil and nifedipine up 200 µM had no effect. We suggest that stimulus-induced calcium conductivity was due to calcium channels operating in the plasma membrane and stimulated (or activated) under conditions when intracellular stores were emptied (see D. Clapham, Nature, 1993; J.W. Putney and G. Bird, Cell, 1993). This suggestion is based on our observations that the ATP/ADP-induced calcium channels in Chang cells are induced also by thapsigargin, caffeine or carbachol. The concentration of thapsigargin required to produce an increase in cellular free calcium was much less than that reported to be required for other cell types (<0.1 µM versus 2.0 µM).

Th-Pos384

THROMBIN-STIMULATED Ca^{2+} INFLUX IN THE HUMAN PLATELET IS A Ca-CALMODULIN DEPENDENT PROCESS SUBJECT TO POSITIVE FEEDBACK. ((W. Watzek, J.G. Cantave, D.H. Haynes)) Dept. M.&C. Pharmacology, Univ. Miami Sch. Med., Miami, FL 33101

The absolute rates of Ca^{2+} influx into Ca^{2+}-depleted human blood platelets were measured by a method based on variable loading with quin-2. The process was measured during the first 10 sec after adding 2 mM external Ca^{2+} to Ca^{2+}-depleted platelets and rates were determined as m mole Ca^{2+}/liter cell volume/min (mM/min). Thrombin-stimulated rates were measured by simultaneously adding 0.5 U/ml thrombin. Calcium-calmodulin and thromboxane A_2 dependence of the processes was assessed by preincubation with the calmidazolium and indomethacin, respectively. The influx rates (mM/min; ave.$\pm$ SD) were:

Thrombin	Calmidazolium	Indomethacin	Rate
0	0	0	0.48$\pm$0.11
0	+	0	0.53$\pm$0.19
+	0	0	1.54$\pm$0.27
+	+	0	0.69$\pm$0.26
+	0	+	1.03$\pm$0.44
+	+	+	0.46$\pm$0.12

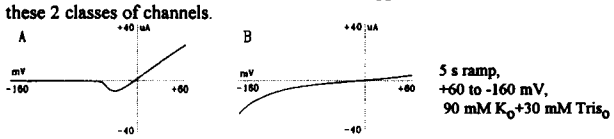
The data show that as much as 80% of the thrombin-stimulated component of Ca^{2+} influx is calmidazolium-inhibitable, that approx. 50% is indomethacin-inhibitable, and that 100% is inhibitable by the calmidazolium/indomethacin combination. This suggests that thrombin-stimulated Ca^{2+} entry is a Ca-calmodulin mediated process subject to positive feedback. Support: FL/AHA and Pharma-Logic, Inc.

NEW K^+ CHANNELS

Th-Pos385

A POSSIBLE STRUCTURAL LINK BETWEEN VOLTAGE-GATED AND INWARD RECTIFIER K^+ CHANNELS. ((J. Tytgat, J. Vereecke and E. Carmeliet)) Laboratory of Physiology, University of Leuven, Belgium.

The body plan of voltage-gated K^+ channels consists of 6 transmembrane-spanning regions, which include a voltage-sensor and a K^+-selective pore, with cytoplasmic amino- and carboxy-terminal domains. The body plan of inward rectifier K^+ channels consists of a K^+-selective pore flanked by 2 transmembrane-spanning regions and cytoplasmic amino- and carboxy-terminal domains. To investigate a possible connection between these 2 classes of K^+ channels, we have deleted transmembrane domains S1 to S4 of a delayed rectifier RCK1 (Kv1.1) K^+ channel and have re-ligated the amino-terminal domain to transmembrane domain S5. Functional expression of the wild-type and mutant channel (whole-cell and single channel data) reveals that the mutant channel is activated upon hyperpolarization, showing a strong inward rectification (B), while the wild-type is activated upon depolarization, showing an outward rectification (A). This phenotypical change associated with the removal of transmembrane-domains S1 to S4 suggests a structural link between these 2 classes of channels.



Th-Pos386

EFFECT OF TEMPERATURE AND INTERNAL MG^{2+} ON INWARD RECTIFICATION OF THE CAT AND GUINEA-PIG CARDIAC INWARDLY RECTIFYING K^+ CHANNEL

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A sarcolemmal ion channel that conducts less current during depolarizations relative to the resting potential than during equivalent hyperpolarizations exhibits the property of inward rectification. In cardiac ventricular myocytes (CVM) the inwardly rectifying K^+ channel, (I_{K1}) contributes importantly to establishing the resting membrane potential, determines the beat rate and the duration of the action potential. Because outward I_{K1} currents could be elicited from guinea-pig CVM at 15° C in the absence but not in the presence of intracellular Mg^{2+} (Mg^{2+}_i), the hypothesis that Mg^{2+}_i mediates the mechanism for inward rectification of I_{K1} emerged and has been extrapolated to include other species. This idea was tested using enzymatically isolated CVM obtained from cat, guinea-pig and rabbit and the whole-cell and excised inside-out patch configurations of the voltage-clamp technique. No Mg^{2+}_i-sensitive outward I_{K1} currents could be elicited from guinea-pig CVM at 37° C, although they could be elicited at 15° C. Thus Mg^{2+}_i appears to have an important role in the inward rectification of guinea-pig cardiac I_{K1} at unphysiologically low temperatures. The fact that the outward currents elicited at reduced temperatures decayed suggests that an additional mechanism may play a role in I_{K1} inward rectification. As temperature was brought to a more physiological level (e.g., 30° C), these Mg^{2+}_i-dependent outward I_{K1} currents from guinea-pig CVM could no longer be evoked. No Mg^{2+}_i-sensitive outward I_{K1} currents could be elicited from cat CVM at 37° C and I_{K1} inwardly rectified. In contrast with the guinea-pig results, independent of Mg^{2+}_i concentration at 15°, 10° and 5° C, I_{K1} remained inwardly rectifying in cat CVM. Rabbit CVM behaved similarly to cat CVM. In general, at physiologically relevant temperatures the mechanism(s) underlying inward rectification of cardiac I_{K1} appears to be Mg^{2+}_i-independent and an obligatory role for Mg^{2+}_i, acting internally to block the I_{K1} channel is not necessary to explain inward rectification.

Th-Pos387

DIFFERENT ACTIONS OF EXTRACELLULAR pH ON TWO CLONED DELAYED RECTIFIER TYPE K^+ CHANNELS, MINI-K AND Kv1.5. ((T. Yamane, T. Furukawa and M. Hiraoka)) Department of Cardiovascular Diseases, M.R.I. Tokyo Medical & Dental University, Tokyo, Japan.

Cardiac delayed rectifier K^+ current is suppressed by external acidification, which might cause electrophysiological derangement during ischemia. In order to explore its molecular basis, we compared modes of proton block on two cloned delayed rectifier type K^+ channels with different structure, Mini-K and Kv1.5. The current (I_{sK} or $I_{Kv1.5}$) was recorded by two microelectrode voltage clamp method from Xenopus laevis oocytes, in which cRNA transcribed from Mini-K or Kv1.5 cDNA was injected. External acidification promptly and reversibly suppressed both I_{sK} and $I_{Kv1.5}$. Although the maximal conductance (G_{max}) was reduced in both cases, block was voltage-dependent only in the case of Kv1.5. While the activation kinetics of Kv1.5 was slowed by external acidification, that of Mini-K was not changed. The activation curve of Kv1.5 was shifted to depolarized direction, which can be well explained by the "surface charge screening model". The Hill coefficient was close to a unity for Mini-K, but was around 2.0 for Kv1.5. Thus, external proton blocked Mini-K and Kv1.5 in totally different manners. Proton binding site for Mini-K was assumed to be outside of the membrane electrical field, whereas those for Kv1.5 were inside of the field, estimated to be 0.31 from the outer membrane surface.

Th-Pos388

THE GUINEA PIG CARDIAC I_{sK} PROTEIN: CLONING AND REGULATION BY PROTEIN KINASE C ((R. Swanson, N.K. Jurkiewicz, Z.J. Zhang, K. Folander, E. Lazarides, and J.J. Salata)) Pharmacology Dept., Merck Research Labs, W. Point, PA

We have isolated cardiac cDNA and genomic clones encoding the guinea pig I_{sK} protein. The deduced amino acid sequence is ~78% identical to the rat, mouse, and human variants of this channel and the structure of the gene encoding the protein is also similar to that in other species. For example, the gene is present only once in the haploid genome, the protein coding sequence is present on a single uninterrupted exon, an intron exists within the 5'-untranslated domain, and multiple alternative polyadenylation sites are used in processing the transcript. Expression of the guinea pig protein in *Xenopus* oocytes results in a slowly activating, voltage-dependent K^+ current, I_{sK}, similar to those expressed previously from the rat, mouse, and human genes. However, in sharp contrast to the rat and mouse currents, activation of protein kinase C with phorbol esters increases the amplitude of the guinea pig I_{sK} current, analogous to its effects on the endogenous I_{Ks} current in guinea pig cardiac myocytes. The phenotype of the current response to phorbol esters can, however, be changed from enhancement to inhibition by mutagenesis to alter 4 cytoplasmic amino acid residues (N102S; C103F; S105A; C107Y). Like the rat and mouse, currents elicited by expression of this quadruple mutant guinea pig channel are inhibited by activation of this kinase. These data explain previously reported differences in the regulatory properties between recombinant rat or mouse I_{sK} channels and native guinea pig I_{Ks} channels and provide further evidence that the I_{sK} protein forms the channels that underlie the I_{Ks} current in the heart.

Th-Pos389

ENHANCEMENT OF THE GUINEA PIG I_{sK} K^+ CURRENT BY PROTEIN KINASE C MEDIATED PHOSPHORYLATION OF SER^{105}

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The I_{sK} protein is thought to form the channels that underlie the slow component of the cardiac delayed rectifier potassium current, I_{Ks}. In guinea pig cardiac ventricular myocytes, activation of protein kinase C with phorbol esters results in an enhancement of this endogenous I_{Ks} current. Similarly, the current resulting from the heterologous expression of the guinea pig I_{sK} protein in *Xenopus* oocytes is also enhanced by application of the phorbol ester PDD (50 nM). The effects of the phorbol esters on the current in oocytes result from activation of PKC since 4αPDD, an inactive isomer, has no effect on the amplitude of I_{sK}. The guinea pig I_{sK} protein has 5 serine residues within its carboxy-terminal, putative cytoplasmic domain (S64, S68, S74, S84, and S105). Each of these Ser residues was individually mutated to Ala, and the mutants expressed in oocytes and assayed for their response to PDD. With the exception of S68A, the magnitudes of the currents expressed by each of the mutants was similar to the wild-type guinea pig I_{sK} current. S68A, however, caused a large apparent shift in voltage dependence to more depolarized potentials, resulting in extremely small currents that were difficult to assay for effects of PDD. Of the other 4 mutations, only S105A changed the phenotype of the current response to PDD. Currents resulting from the expression of S105A were unaffected by activation of PKC whereas the responses of the S64A, S74A, and S84A were increased by PDD in the same manner as the wild-type. The data demonstrate that the enhancement of guinea pig I_{sK} currents by phorbol esters results from PKC-mediated phosphorylation of Ser^{105} in the channel.

Th-Pos390

THE I_{sK} PROTEIN IS A TYPE III INTEGRAL MEMBRANE PROTEIN

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The I_{sK} protein forms the channels that underlie the slow component, I_{Ks}, of the cardiac delayed rectifier potassium current, I_K. This protein consists of only 125-130 amino acids, encoding a single hydrophobic, putative transmembrane, domain and is structurally unrelated to subunits of most other known ion channels. Studies have been undertaken to determine the topology of this protein within the membrane. The amino terminal, hydrophilic domain of the protein has been determined to be extracellular by: 1) immunological localization of an NH_2-terminal epitope tag to the extracellular space, 2) identification, by site-directed mutagenesis, of the protein's extracellular La^{3+} binding site to specific residues within this domain, and 3) demonstration, by both mutagenesis and enzymatic deglycosylation, that the protein contains N-linked sugars at residues Asn^5 and Asn^{26}. The C-terminal hydrophilic domain has been located cytoplasmically by 1) immunological localization with an anti-peptide serum that specifically recognizes a carboxy-terminal epitope in the protein and 2) mutagenesis to identify PKC substrate sites within this domain. Together, the data demonstrate that the protein has an extracellular N-terminus, a single transmembrane domain, and an intracellular C-terminus. With this topology, and the lack of a canonical signal sequence, the I_{sK} protein is classified as a type III integral membrane protein (von Heijne classification). Although proteins of this class are unusual, the influenza virus M2 protein, which forms monovalent ion channels, is another example. Thus, the I_{sK} protein may be one representative of a functionally diverse but structurally similar family of ion channels.

Th-Pos391

VOLTAGE-DEPENDENCE OF DEACTIVATION OF I_{SK} SUGGESTS A SUBUNIT ASSEMBLY MODEL FOR CHANNEL GATING.

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The voltage-dependent potassium channel min K encodes a protein with a molecular structure very different from other voltage-gated potassium channels. The min K protein has been cloned from many species, most recently guinea pig (gpmin K), and is thought to be responsible for the slowly activating cardiac potassium current I_{Ks} (Varnum et. al., 1993, PNAS, in press). When expressed in *Xenopus* oocytes, the characteristic feature of gpmin K is a very slowly activating potassium current, I_{SK}, with activation rates on the order of seconds in contrast to msec activation rates for *Shaker*-like potassium channels. A kinetic model for min K has not been proposed perhaps because of its unusually slow kinetics of activation. Analysis of activation and deactivation rates of I_{SK} at 22 °C reveal that deactivation is approximately 4 to 6-fold faster than activation at the same voltage. The rate of deactivation becomes voltage-independent at extreme negative potentials, approaching a limiting-time constant of approximately 500 ms. This data, plus the finding that chemical crosslinking agents induce persistent activation of I_{SK}, suggest a fundamentally different gating mechanism. We interpret our findings with a kinetic scheme in which: (1) individual min K subunits may be localized in restricted sites in the membrane but are in random motion, (2) a voltage-dependent conformational change of individual min K subunits allows subunits to assemble when a collision between activated subunits occurs and (3) following assembly of n (unknown) subunits a potassium selective channel is formed. This scheme predicts that the rate limiting step for deactivation would be the dissociation of subunits. A rate-limiting step with a time constant of 500 ms for deactivation places an upper limit of 2 s^{-1} for dissociation of subunits.

Th-Pos392

CLONING AND FUNCTIONAL EXPRESSION OF A RAT HOMOLOGUE OF THE VOLTAGE GATED EAG ION CHANNEL.

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The analysis of behavioural mutants of *Drosophila melanogaster* has led to the identification of several potassium channel families: *Shaker*, *slowpoke* and *ether à gogo (eag)*. While *Shaker* and *slowpoke* represent families of voltage-gated and calcium-activated potassium channels, respectively, *eag* exhibits sequence similarities not only to potassium channels but also to cyclic nucleotide-gated cationic channels. Functional expression in *Xenopus* oocytes revealed that the *eag* polypeptide forms a new type of voltage-gated channel that is permeant for potassium and calcium and modulated by cyclic nucleotides.

We now have cloned a cDNA from rat brain coding for a vertebrate potassium channel subunit (reag) that is homologous to *eag*. The deduced amino acid sequence shares 65 % identical residues with the *Drosophila* polypeptide in the N-terminal region comprising six putative transmembrane segments, the pore and a binding site for cyclic nucleotides. Northern blot analysis and *in situ* hybridization studies show that reag transcripts are almost exclusively localized in the brain and central nervous system. Injection of in vitro transcribed reag-cRNA into *Xenopus* oocytes leads to the expression of voltage gated potassium channels with pharmacological profile similar to *eag*. The sequence of permeabilities differs to eag in that reag is not permeable to Cs ($K>Rb>NH_4>Li\sim Na\sim Cs$). Further data on the electrophysiological properties of reag channels will be presented.

Th-Pos393

ETHER-À-GO-GO: A POTASSIUM AND CALCIUM PERMEABLE CHANNEL MODULATED BY CAMP

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Ether-à-go-go (*eag*) are *Drosophila melanogaster* mutants that show behavioural abnormalities under ether anaesthesia. It has been shown that in larval muscle of *eag* mutants several K^+ currents are altered, including fast inactivating, delayed rectifier and two Ca^{2+}-dependent K currents[1]. The *eag* gene encodes a polypeptide that shares sequence similarities with the family of voltage-gated ion channels as well as with the cyclic nucleotide-gated channels, containing a putative cyclic nucleotide binding domain[2].

We have expressed *eag* cRNA in *Xenopus* oocytes and found that it functionally expresses a channel permeable to both K^+ and Ca^{2+}. The sequence of permeabilities is $K^+>Rb^+>Cs^+>NH_4^+>Na^+>Li^+$ which correspondes to the Eisenman's series IV. The kinetics observed in inside-out patches show a fast inactivating component as well as a non-inactivating component. In addition, the current is directly modulated by cAMP and indirectly by cGMP.

Taken together, these properties are able to explain the electrophysiological alterations seen in the mutant larval muscle. We think that *eag* is implicated in the presynaptic facilitation mechanism observed at the neuromuscular junction of *Drosophila melanogaster*.

1. Zhong, Y. and Wu, C.-F. *Science* 252, 1562-1564 (1991)
2. Warmke, J., Drysdale, R. and Ganetzky, B. *Science* 252, 1560-1562 (1991)

Th-Pos394

STRUCTURE FUNCTION STUDIES OF IONIC PERMEABILITY AND cAMP MODULATION OF DROSOPHILA EAG.

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The *ether à-go-go* (*eag*) mutation in *Drosophila* confers repetitive firing of action potentials in motor axons and abnormal release of transmitter at the larval neuromuscular junction. Voltage clamp analysis of *Drosophila* larval muscles has revealed that several K^+ currents are affected in *eag* mutants (Zhong and Wu, Science 252, 1562, 1991). The *Xenopus* oocytes expression of *eag*-encoded cDNA gives rise to a voltage dependent non-inactivating K^+ current with a detectable permeability to Ca^{2+} ions and is modulated by cAMP (Brüggemann *et al.*, Nature, 365, 445, 1993). So this channel shares the characteristics of both voltage and ligand activated channels, as can be predicted from the nucleotide sequence of *eag*.

Here we describe the effects of site directed mutagenesis in both the pore region and the putative cyclic nucleotide binding region of *eag* by electrophysiological measurements of the expressed channels in *Xenopus*. Mutating a single amino acid residue in the C-terminal half of H5 alters both the kinetics and the permeability of the channel. Further mutants in the H5 region are under investigation. Also, a single point mutation in the carboxy terminus of *eag* makes the channel insensitive to cAMP. This result, in combination with the ones of patch clamp experiments, indicate that cAMP modulates *eag* by direct binding to a cyclic nucleotide binding pocket located within the carboxy terminus.

Th-Pos395

CHARACTERIZATION OF AN EXPRESSED INWARD RECTIFIER POTASSIUM CHANNEL:THE EFFECT OF P-LOOP MUTATIONS

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In an effort to understand the molecular mechanism of rectification of the inward rectifier K^+ channel (IK1) we characterized IK1 cloned from the mouse macrophage cell line J774 (Kubo *et al.*, 1993) and several pore mutants. Mutations were selected based on comparison of P-Loop sequences of inwardly rectifying K^+ channels. The channel variants were expressed in *Xenopus* oocytes and studied utilizing two-microelectrode voltage clamp and patch clamp recording. At the whole-cell level the wild-type channel exhibited time- and voltage dependent block of inward current by Na^+ and Ba^{2+}. At the single-channel level the γK^+ was 25 pS (96 mM K^+_{out}) and increased to 45 pS in the presence of EDTA. Inside-out patches excised into Mg^{2+}-free bath solution demonstrated no change in inward rectification, but significant current rundown. Neutralization of the glutamate at position 138 (E138T,V) eliminates ionic conductance. Replacement of the glutamine at position 140 with methionine (Q140M) produces a channel with wild-type behavior with respect to inward rectification and block by external cations. We conclude: 1. The expressed wild-type IK1 and Q140M mutant are blocked in a voltage-dependent fashion by external cations and demonstrate Mg^{2+}-independent inward rectification. 2. The pore of the channel is sensitive to site-specific changes.

Th-Pos396

GENOMIC STRUCTURE AND CHROMOSOMAL MAPPING OF THE INWARDLY RECTIFYING K+ CHANNEL IRK1. ((J. Redell and B. Tempel)) Dept. of Pharmacology, Univ. of Washington, Seattle, WA. 98195. (Spon. by T. Hinds)

Inwardly rectifying K+ channels are not gated by membrane potential, but conduct an inward K+ current at hyperpolarizing membrane potentials. Their inward rectification arises from an internal magnesium block that inhibits K+ efflux at depolarized membrane potentials. Because of their rectification properties, these channels play an important role in regulating the resting membrane potential and electrical excitability of cells in a variety of tissues, including brain and heart. Recently, the cDNA expression cloning in *Xenopus* oocytes of three inwardly rectifying channels: ROMK1 (Nature 362:31), IRK1 (Nature 362:127), and GIRK1 (Nature 364:802), has allowed a look at the primary structure of these channels. In contrast to the 6-transmembrane and H5 pore structure of the voltage-gated K+ channel family, these inward rectifiers display a 2-transmembrane and H5 pore structure. The cloning of these channels has provided us with the opportunity to define their chromosomal loci and investigate their genomic structures. Utilizing an open reading frame probe derived from the IRK1 cDNA, we screened a mouse strain 129 λ phage genomic library at high stringency and obtained 18 independent isolates. Restriction analysis of 9 purified isolates allowed their classification into 5 overlapping groups of clones spanning a total of approximately 23-28 Kb of genomic DNA, with each group containing the entire IRK1 ORF. Alignment of cDNA and genomic clones by restriction mapping and PCR analysis indicates that, similar to several murine *Shaker*-like K+ channel genes, the ORF of IRK1 is intronless. Preliminary analysis of the 3' and 5' UTRs indicates that the 3' end is contiguous with the ORF and intronless, while the 5' UTR apparently contains at least 1 intron. We are currently attempting to locate and analyze the 5' transcriptional start site. The chromosomal location of the IRK1 gene is being analyzed by fluorescent *in situ* hybridization to murine and human chromosomes, as well as being mapped in a *M. musculus/M.spretus* interspecific backcross panel using an identified restriction fragment length polymorphism.

Th-Pos397

CHEMICAL AND ELECTROCHEMICAL CONTROL BY K^+ ITSELF, IN GATING THE MAJOR OUTWARD-RECTIFIER K^+ CHANNEL (YPK1) OF *SACCHAROMYCES* ((Adam Bertl and Clifford L. Slayman)) Pflanzenphysiologisches Institut, Universität Göttingen, Germany; and Department of Cellular and Molecular Physiology, Yale University, New Haven CT, USA (Sponsored by J.F. Hoffman).

The dominant outward-rectifying potassium channel in yeast plasma membrane displays three distinct and parallel modes of gating: from a single Open state, to long quiet Gaps, to quick Interrupts which appear as open-channel noise, and to slower Ca^{++}-induced Blocks. Under the usual recording conditions (~200 mM cytoplasmic-side KCl, 100 μM $CaCl_2$ / 50-100 mM outside KCl), the channel open probability (P_o) is appreciable (> 0.1) only at positive membrane voltages (V_m), peaking at ca. +100 mV. At physiologic $[Ca^{++}]_{cyt}$ (~300 nM), open probabilities for normal negative V_m are below 0.005. Such properties would imply, but not identify, very specialized physiologic functions for YPK1 channels (*J. Membr. Biol.* 132: 183-199, 1993).

However, more relaxed functions would be possible if gating were modulated by the net *electrochemical* potential for K^+ ($\Delta V_K = V_m - E_K$), rather than strictly by V_m, as discussed earlier by Ciani et al. (*J. Membr. Biol.* 44:103-134, 1978). We have therefore examined the dependence of P_o upon K^+ concentration, both $[K^+]_{cyt}$ and $[K^+]_{out}$. Of the six first-order rate constants involved in the above gating model, three proved K^+-insensitive: k_{OB},k_{BO}, and k_{OI}; two are proportional to $[K^+]_{cyt}$: k_{IO}, and k_{GO}; and one, k_{OG}, is proportional to $[K^+]_{out}$. Thus P_o can *appear* to depend upon ΔV_K, at least for the **O** ↔ **I** and **O** ↔ **G** transitions, but the dependence is simple only if concentration changes are restricted to one side of the membrane.

Th-Pos398

FUNCTIONAL CHARACTERIZATION OF A CLONED HUMAN KIDNEY INWARDLY RECTIFYING K^+ CHANNEL.

((Kugler, J.L., Yano, H., Takeda, Tokuyama, Y., J., Nelson, D.J., And Philipson, L.))The University of Chicago, Chicago, Illinois, 60637 USA

Recent studies have identified a new family of K^+ channels, which differ from the voltage gated channels in that they have inwardly rectifying current-voltage relationships. They are also believed to have only two transmembrane segments which have limited homology to the fifth and sixth transmembrane domains of the voltage gated channels, as well as a region which is clearly related to the putative pore-forming region of the larger channels. One inwardly rectifying K^+ channel (Ho et al, 1993; ROMK1) was isolated by expression cloning from rat kidney. We have employed a polymerase chain reaction based strategy to isolate two human kidney ROMK1 cDNA clones derived from three different mRNAs differing in sequences at the 5' end. Characterization of the human ROMK1 gene indicates that this diversity is generated by alternative splicing. Human ROMK1A mRNA encodes a protein (hROMK1A) of 389 amino acids, with 93% identity to the 391 amino acid rat homologue. The sequences diverge at the N-terminus as well as at several potential phosphorylation and glycosylation sites. hROMK1B and hROMK1C mRNAs are predicted to code for a 372 amino acid protein which is "truncated" at the N-terminus. RNA blotting studies revealed a 4 kb transcript, present in kidney and absent in heart, lung, pancreas, liver, skeletal muscle, spleen and placenta. RT-PCR analysis revealed the presence of hROMK1A mRNA in pancreatic islets. Two electrode voltage clamp of *Xenopus* oocytes microinjected with RNA coding for hROMK1A revealed extracellular currents similar to those of ROMK1 in inward rectification and sensitivity to block by extracellular cations. Reversal potential of barium sensitive currents varied directly with external potassium concentration, indicating high potassium selectivity. This suggests that the variations in sequence between ROMK1 and hROMK1A do not markedly affect the parameters studied thus far. Detailed characterization of these and other members of the new inward rectifier superfamily will further our understanding of molecular structure-function and evolutionary relationships among these channels.

Th-Pos399

MOLECULAR RECOGNITION BETWEEN MEMBRANE-LOCATED SYNTHETIC SEGMENTS OF K^+ ION CHANNELS. ((Y. Shai and I. Ben-Efraim)) Dept. of Membrane Research and Biophysics. Weizmann Institute of Science, Rehovot, 76100 Israel. (Spon. by K. Rosenheck)

Current models of inwardly rectifying K^+ channels, namely: ROMK1, IRK1 and GIRK1 predict that the channels are formed by the coassembly of four polypeptide monomers, each of which consists of 2 trans-membrane segments (M-1 and M-2) and long terminal domains. The aqueous pore is proposed to be composed of the conserved H-5 regions contributed by four monomers. We have synthesized and fluorescently labeled peptides corresponding to the putative trans-membrane M-2 and the H-5 region, and structurally and functionally characterized them. The data reveal that: (i) only M-2 adopts α-helical structure (73%) in hydrophobic environment, (ii) both segments bind to zwitterionic phospholipid vesicles, (iii) both peptides self-associate in their membrane-bound state and (iv) membrane located H-5 associates with membrane-bound M2 but do not associate with unrelated membrane-bound α-helical peptides. These data combined with similar properties assigned to the H-5 and the S-2 regions of the more structurally complex *Shaker* potassium channel (Peled, H., & Shai, Y. (1993) Biochemistry 32, 7879-7885; Peled, H., & Shai, Y. (1993) Workshop on Molecular Basis of Ion Channel Function. Madrid, Spain) demonstrate recognition between transmembrane segments of K^+ channels. Such recognition might contribute to the oligomerization and to the correct assembly of the monomers to form a functional channel.

Th-Pos400

ACTIVATION OF ATP-SENSITIVE K^+ CHANNELS AND CELLULAR K^+ LOSS.((N. Deutsch, L.D. Alexander, P. Shang and J.N. Weiss)) UCLA School of Medicine, Los Angeles, CA, 90024.

The role of the ATP-sensitive K^+ current ($I_{K,ATP}$) during myocardial hypoxia and ischemia in causing net cellular K^+ loss is controversial. We exposed isolated arterially perfused rabbit intraventricular septa loaded with ^{42}K to 12 μM cromakalim (CK) to activate $I_{K,ATP}$ and measured the effects on APD, unidirectional K^+ efflux rate and net tissue K^+ content. The results were compared to 30 min of substrate-free hypoxia. Both CK and hypoxia caused a similar degree of APD shortening (to 37 ± 7% and 41 ± 8% of control after 10 min, respectively) and increase in unidirectional K^+ efflux rate (from 1.24 ± 0.11 to 1.75 ± 0.18 μmol/g-/min (n = 6) during CK, and from 1.36 ± 0.16 to 1.95 ± 0.22 μmol/g/min (n = 5) during hypoxia). Net tissue K^+ content after 30 minutes did not change during CK (from 22.9 ± 1.2 to 22.9 ± 1.2 μmol/g, n = 4), but decreased significantly during hypoxia (from 23.7 ± 0.6 to 18.1 ± 1.5 μmol/g, n = 4, $p < 0.05$). Pre-treatment with 100 μM glibenclamide (GB) blocked most of the increase in K^+ efflux during hypoxia (from 1.04 ± 0.12 to 1.18 ± 0.14 μmol/g/min (n = 5)).The decline in tissue K^+ content during hypoxia was partially blocked by GB (from 21.1 ± 0.6 to 18.7 ± 1.5 μmol/g, n = 4, $p < 0.05$ compared to hypoxia alone). During myocardial hypoxia or ischemia, activation of $I_{K,ATP}$ alone is not sufficient to cause net cellular K^+ loss and other factors, perhaps a concomitant increase in inward currents, may be required.

Th-Pos401

REGULATION OF ATP-SENSITIVE K^+ CURRENT BY LEVCROMAKALIM AND NUCLEOTIDES IN RABBIT PULMONARY ARTERY.
((L.H. CLAPP)) Cardiovascular Research, UMDS, St. Thomas's Hospital, London SE1 7EH, U.K.

Substantial evidence suggests that ATP-sensitive K^+ channels (K_{ATP}) are activated by K^+ channel opening drugs (KCOs) such as levcromakalim, although in vascular smooth muscle little is known about the regulation of this channel. Using enzymatically isolated cells from rabbit pulmonary artery, whole-cell voltage-clamp recordings have been made of holding current at -60 mV using symmetrical 139 mM K^+ solutions. With 1 mM ATP and 500 μM GTP in the pipette, the glibenclamide-sensitive (10 μM) holding current (I_{glib}) was small (-10 ± 4 pA; mean ± s.e.m., n=13), and bath application of 10 μM levcromakalim activated an inward current (I_{lev}) of -108 ± 14 pA (n=24). Lowering intracellular ATP to 100 μM significantly increased ($p<0.01$) both the magnitude of I_{glib} (-59 ± 15 pA, n=10) and I_{lev} (-235 ± 36 pA, n=13). However, omitting GTP from the pipette reduced I_{glib} to -24 ± 6 pA (n=10, $p<0.05$) while having no effect on I_{lev}, such that after 28 mins of recording, I_{lev} was still -255 ± 125 pA (n=5). Furthermore, with 500 μM GDP and 100 μM ATP in the pipette, I_{glib} was larger (-114 ± 19, n=3) compared to GTP, while I_{lev} was reduced (-69 ± 19 pA, n=3). These results suggest that intracellular ATP modulates the response to KCOs, and that guanosine nucleotides, whilst not a prerequisite for the action of these drugs, can also activate K_{ATP} channels.

Supported by the Wellcome Trust

Th-Pos402

AN ATP-SENSITIVE, SULFONYLUREA-INHIBITABLE K^+ CONDUCTANCE IN NATIVE RABBIT ARTERIAL ENDOTHELIAL CELLS.
((C. Katnik & D.J. Adams)) Dept. of Molecular & Cellular Pharmacology, Univ. of Miami School of Medicine, Miami, FL.

Arterial endothelium regulates the tone of vascular smooth muscle by releasing endothelium-derived relaxing and contracting factors, a process that has been linked to changes in endothelial cell $[Ca^{2+}]_i$ and membrane potential. Conditions that induce hypoxia, such as ischemia-reperfusion, have been found to deplete ATP levels in arterial endothelial cells (Arnould *et al.*, 1992, *J. Cell. Physiol.* 152:215-221). We have investigated the electrophysiological and pharmacological properties of an ATP-sensitive K-conductance (K_{ATP}) in endothelial cells freshly dissociated from rabbit aorta. The presence of K_{ATP} channels in vascular endothelial cells was determined using the following criteria: current activation by $[ATP]_i < 1$ mM or by the K-channel opener levcromakalim (BRL 38227) and inhibition of these currents by the sulfonylurea drugs, glibenclamide or tolbutamide (Edwards & Weston, 1993, *Ann. Rev. Pharmacol. Toxicol.* 33:597-637). Bath application of levcromakalim (3 μM), or the metabolic inhibitors dinitrophenol (50 μM) and iodoacetic acid (1 mM) produced an increase in whole-cell membrane currents measured in symmetrical K^+ solutions (140 mM). Under current clamp conditions, K_{ATP} current activation hyperpolarized the cell ~20 mV from the resting membrane potential (-40 mV) in 140 mM NaCl bath solutions. These currents were reversibly inhibited by glibenclamide (IC_{50}=42 nM) and tolbutamide (1 mM), as well as by external Ba^{2+} (IC_{50}=1.1 mM), but were insensitive to charybdotoxin (100 nM) and TEA (5 mM). In response to voltage ramps from -140 to -40 mV, in symmetrical KCl, the whole-cell currents activated by levcromakalim were linear, had a slope conductance of ~30 pS/pF and exhibited a cation selectivity sequence: $K^+ = Rb^+ > Cs^+ > > Na^+ > NH_4^+ > Li^+$. Using internal perfusion of the pipette and cell, an increased membrane current was observed in the absence but not the presence of 3 mM Mg-ATP in the patch pipette. These results indicate that reduction of intracellular [ATP], metabolic inhibition or exposure to levcromakalim activates a K^+ conductance that modulates endothelial membrane potential and may influence vascular tone.

Th-Pos403

KINETICS OF PINACIDIL-INDUCED I_{KATP} ARE MODIFIED BY INTRACELLULAR ATP.
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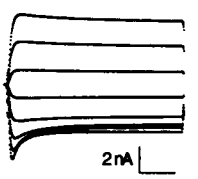
We have investigated the time course of pinacidil-induced current using whole-cell and single channel recordings in guinea-pig ventricular myocytes. Under whole-cell patch clamp configuration, in the presence of $[ATP]_i \geq 1.0$ mM, pinacidil (200 μM) has previously been shown to induce time-independent outward current positive, but not negative, to E_K. This has been identified as the ATP-sensitive potassium current (I_{KATP}). In contrast, we now find that when cells are dialyzed with solutions containing nominally zero ATP, the current induced by pinacidil is time-dependent in the inward direction, showing a rapid peak that subsides within 50 ms (figure). The time constants were 1.5±0.1 ms and 7.5±1.2 ms at -130 mV from a holding potential of -40 mV, with $[K^+]_o$=5 mM. It is sensitive to and inhibited by glibenclamide (200 nM). In cell-attached patch condition using $[K^+]_o$=5mM, we find ensemble patch currents that display qualitatively similar time course. However, in either cell-attached or excised patch conditions in which symmetric 140 mM $[K^+]$ and $[ATP]_i \geq 50$ μM were used, ensemble patch pinacidil-induced current did not rectify and was time-independent. Our results suggest that both K_o^+ and ATP_i interact to cause rectification of macroscopic current induced by pinacidil in heart.



Th-Pos404

INTERACTION BETWEEN THE ACTIVATION OF I_{K1} AND $I_{K\text{-}ATP}$ CHANNELS IN GUINEA-PIG VENTRICULAR MYOCYTES.
((Stephane N. Hatem, Dominique Rooney, Martin Morad)) Dept. of Pharmacology, Georgetown University, Washington, D.C. 20007

The effects of various intracellularly applied nucleotides on the inward rectifier K^+ current (I_{K1}) during prolonged phosphorylation were examined in whole cell-clamped guinea-pig ventricular myocytes. Within 5 minutes of addition of forskolin (1 μM), in myocytes dialyzed with internal solution containing either GTPγS, GDPβS, UTP, ADTβS, GTP, or GDP, a large voltage-independent potassium current was activated which decayed spontaneously in 3-4 minutes. Glybenclamide (10μM), which had no effect on I_{K1} current under control conditions, rapidly and fully blocked the forskolin-activated current. The spontaneous decay of forskolin-activated current led also to the complete disappearance of I_{K1}, such that no voltage activated K^+ current could be recorded even at very negative potentials. In this state, $I_{K\text{-}ATP}$ channel openers (lemakalin and ER 001533) failed to activate the $I_{K\text{-}ATP}$ channel. The Forskolin-activated current and complete rundown of I_{K1} were not caused by changes in [ATP]$_i$ or [ADP/ATP]$_i$ ratio since dialyzing the myocytes with an internal solution containing 5 mM of fresh ATP plus an ATP regenerating system, creatine-phyosphate and creatine phosphokinase, did not alter the time course of activation of the forskolin-activated current nor did it alter the decay of I_{K1}. The activation of the forskolin-activated current was suppressed by dialyzing the myocytes with the amino-acid fragment of the cAMP-dependent protein kinase inhibitor indicating that this effect resulted from the activation of protein kinase A. We conclude that the burst like appearance of a glybenclamide-blockable current following prolonged exposure to forskolin and subsequent decay, may represent activation of $I_{K\text{-}ATP}$ channel. The disappearance of I_{K1} channel simultaneous with the activation of $I_{K\text{-}ATP}$-like channel suggests possible "cross-talk" in the activation and regulation of these two K^+ channels.

Th-Pos405

MECHANISM OF INTRACELLULAR DI- AND TRI-VALENT CATION INHIBITION OF ATP-SENSITIVE K CHANNELS IN RAT VENTRICULAR MYOCYTES.
((Z. Fan & J.C. Makielski)) *Univ. of Chicago, Chicago, IL*

Intracellular divalent cations (Ca^{2+}, Mg^{2+}) are known to decrease open probability (P_o) of ATP-sensitive K channels (K.ATP), but the precise mechanism is unknown. We used single channel recordings in 60 inside-out patches to study K.ATP in symmetrical K^+ solutions in the absence of internal ATP at V_m from -50 to +50 mV and 23 °C. Di- and trivalent cations (0.01 μM to 1.0 mM) added to the cytoplasmic side of the membrane reduced P_o in a time-dependent fashion. After 2 min exposure, the sequence for reducing P_o was $Gd^{3+} \geq La^{3+} > Zn^{2+} \geq Ca^{2+} > Mn^{2+} \approx Mg^{2+}$. This sequence is consistent with an oxygen-ligand rich cation-binding site. The onset of the reduction was more rapid and less reversible for cations to the left of the sequence. For example, 0.1 μM Gd^{3+} reduced P_o to 10%, and the effect was irreversible, while 0.1 mM Mn^{2+} reduced P_o to 90%, and the effect was reversed upon washing. For Gd^{3+} at <1 μM, the effect was the same for -50 mV and +50 mV suggesting no contribution of the membrane field. Exposure to trypsin (2 mg/ml) for >8 min greatly slowed and diminished the effect of Gd^{3+} and virtually eliminated the effect of Ca^{2+} and Mn^{2+}. Exposure to a carboxyl-specific reagent TMO (30-60mM) for 3-10 min failed to prevent the inhibitory effect of Gd^{3+} (n=4). TMO itself induced irreversible inhibition of K.ATP in 10 out of 14 patches. These results suggest 1) a multivalent cation-binding regulatory site on cardiac K.ATP or one of its subunits that is likely to be 2) at the cytoplasmic side, 3) outside the membrane field, 4) possibly composed of the oxygen-ligands. Decreased P_o of K.ATP by Ca^{2+} and Mg^{2+} may play a role in the electrophysiology of Ca^{2+} overload states such as the latter stages of ischemia, reperfusion, or glycoside toxicity. In addition, these findings have implications for the primary channel structure responsible for this multivalent effect.

Th-Pos406

GATING MECHANISMS OF ATP-SENSITIVE POTASSIUM CHANNELS: IMPLICATION IN ISCHEMIA/REPERFUSION INJURY AND AGING.
((C.Y. Lee)) Chemical Dynamics Corp., Guilderland, NY 12084.

It has been well established that oxygen free radicals are generated upon reperfusion or reoxygenation after ischemia in the heart or brain. The ATP-sensitive K channel is known to play important roles during ischemia (Cardiovasc. Res., 26, 1011 (1992)), but how the channel may be involved in the generation of oxygen radicals is not clear. We propose that, similar to the Shaker K channels [Lee, FEBS Lett., 306, 95-97 (1992)], the ATP-sensitive K channel could be gated by the electron transfer between two stable sites. At the physiological ATP levels, electron transfer is inhibited by the negatively charged ATP. During ischemia, dissociation of ATP from the channel induces electron transfer to the site near intracellular surface. Upon reperfusion or reoxygenation, the oxygen molecule may get close to this site to capture an electron, thereby generating the oxygen radical. This mechanism of oxygen radical production is similar to that observed in another well-established electron transfer system [Stadtman, Science, 257, 1220-1224 (1992)], which is involved in the aging process. The reduced levels of ATP also contribute to aging [Beal et al., TINS, 16, 125-131 (1993)].

Th-Pos407

MAGNESIUM BLOCK OF ATP-DEPENDENT POTASSIUM CHANNELS FROM RAT ISOLATED SKELETAL MUSCLE FIBRES. ((H-C. M^cKillen.)) Ion Channel Group, Dept. of Cell Physiology & Pharmacology, University of Leicester, Leicester, England LE1 9HN.

The application of Mg^{2+} to the internal face of patches excised from sarcolemmal vesicles of rat isolated skeletal muscle had two effects on K_{ATP} channel activity. Firstly, it produced a flickery block of outward current and secondly it reduced the channel P_{open} with a Kd (at 0mV) of 2mM and integer Hill coefficient of 2. Channel block was measured with beta-fits and from the fractional unitary current remaining in the presence of Mg^{2+} obtained from ensemble average I-V ramps (Yellen, 1984). Both methods of analysis yielded similar results.
The Mg^{2+} block increased with increasing membrane potential. The relationship between fractional current and $[Mg^{2+}]$ was best fit by a curve with Kd of 5.65mM at 0mV and 1.05mM at +80mV ($[K^+]_o/[K^+]_i$ = 10/155mM, data from 6 patches). The Kd values were plotted against holding potential and a best fit to the Boltzman expression was obtained with an effective valency for the block of 0.61 (from ramp and beta-fit data). This was mainly due to a decrease in the unblocking rate with increasing holding potential.
The block was relieved by increasing the $[K^+]_o$. At +40mV the Kd obtained from the fit to the dose-response curve was 2.05mM in 10mM K^+_o and 7.54mM in 155mM K^+_o (data from 4-6 patches). This was due to an increase in the unblocking rate as $[K^+]_o$ was increased.
The presence of Mg^{2+} in the internal solution had no effect upon the channel affinity for ATP, the Ki value being 15 μM compared to 19 μM measured in other patches in the absence of Mg^{2+}.

Yellen, G. (1984). J. Gen. Physiol. 84,157-186.

Th-Pos408

CHARACTERIZATION AND PARTIAL PURIFICATION OF K^+ CHANNELS EXTRACTED FROM SUBMITOCHONDRIAL PARTICLES. ((Y. Lu and A.D. Beavis)) Dept. of Pharmacology, Med. Coll. of Ohio, Toledo, OH 43699. (Sponsor, A. Askari)

Previously, it has been shown that intact mitochondria possess a uniport pathway for K^+ and a protein mediating K^+ uniport has been partially purified. In this communication we present the results of a study in which proteins solubilized from submitochondrial particles with 3% Triton X-100 have been reconstituted into proteoliposomes and fused with planar lipid bilayers. Using this technique, we observe two distinct K^+ channels, which, under gradient conditions, 600 mM KCl (cis) and 60 mM KCl (trans) have conductances of 210-260 pS and 60-80 pS. The permeability ratio P_K/P_{Cl} for the larger channel is 9 and the conductance increases as the holding potential (cis-trans) becomes more negative. In addition, both the mean open time and mean closed time decrease quite dramatically (>200 ms → 8 ms) as the holding potential is varied from -180 mV to +120 mV. At 59 mV the open probability is close to 90%. The smaller channel has quite different properties. The permeability ratio P_K/P_{Cl} is higher, close to 25. The mean open time is short (3 ms) at all voltages and the open probability appears to be voltage dependent. At 59 mV, we have observed open probabilities of about 2.5% and 12%, depending on the orientation of the channel in the membrane. Following fractionation of the solubilized proteins on a DEAE-cellulose column, both channels are observed in the fraction eluted with 200 mM KCl. Further fractionation of this sample on a mercurial Affigel column fails to separate the channels which are eluted with mercaptoethanol. This work was supported by NIH grants HL 36573 and HL/GM 47735.

MODULATION OF CHANNELS

Th-Pos409

MAITOTOXIN (MTX) ACTIVATES A NON-SELECTIVE CATION CHANNEL (NSCC) PRIOR TO CALCIUM ENTRY.
((P. Dietl and H. Völkl)) Department of Physiology, University of Innsbruck, Austria.

We examined the mechanisms of MTX to stimulate Ca^{2+} entry into a renal epithelial cell (MDCK). In the presence of bath Ca^{2+}, MTX (3 nM) caused an elevation of $[Ca^{2+}]_i$, which was partially inhibited by SK&F 96365 (25 μM) or La^{3+} (100 μM) and coincided with the stimulation of Ca^{2+}-dependent K^+ channels. Prior to the rise of $[Ca^{2+}]_i$, a non-selective cation current (I_{ns}) was irreversibly activated. I_{ns} poorly discriminated between Na^+, K^+ and Cs^+. It was not voltage-gated and not inhibited by nifedipine (10 μM). La^{3+} (100 μM), but not SK&F 96365 (25 μM), partially inhibited I_{ns}. The activation of I_{ns} by MTX was instantaneous and depended on the presence of extracellular Ca^{2+} ions. Cell-attached and excised single channel measurements revealed that MTX activated a ≈ 40 pS NSCC from the outside. We conclude that the initial action of MTX is the stimulation of a NSCC, which requires the presence of extracellular Ca^{2+} ions. The subsequent rise of $[Ca^{2+}]_i$ is at least in part caused by another, SK&F 96365-sensitive Ca^{2+} entry pathway, which may be activated as a result or independently of I_{ns}. This work was supported by the FWF, grant P9101-Med and the Legerlotz-Stiftung.

Th-Pos410

ACETYLCHOLINE PRE-CONDITIONING STIMULATES ACETYLCHOLINE TO ACTIVATE A GLIBENCLAMIDE-SENSITIVE K CURRENT DEPENDENT ON SR Ca^{2+} RELEASE AND PKC.
((Y.G. Wang and S.L. Lipsius)) Loyola University Medical Center, Dept. of Physiology, Maywood, IL 60154

A nystatin-perforated patch whole-cell method was used to study the effects of acetylcholine (ACh) pre-conditioning on ACh-activated K currents in cat atrial myocytes. An initial 4 min. ACh exposure (ACh-1) was followed by a 4 min. recovery period and a second 4 min. ACh exposure (ACh-2). Voltage clamp ramps (40 mV/s) between -130 and +30 mV were used to assess total membrane conductance. At 10 μM, ACh-2 increased K conductance significantly more than ACh-1 and the effect was greater at more negative voltages. Currents elicited by ACh-1 and ACh-2 at -130 mV were -19.4 ±1.5 and -30±1.9 pA/pF, respectively (+58%;P<.002), and at +30 mV were 13.6±1.0 and 18.9±1.5 pA/pF, respectively (+39%;P<.005). K current potentiated by ACh-2 was selectively abolished by: i) 1 μM ryanodine, ii) 30 s exposure to 10 mM caffeine prior to ACh-2, iii) zero external Ca^{2+}, iv) dialysis with 10 mM EGTA, v) 50% external Na, vi) 0.1 μM calphostin C, vii) 10 μM glibenclamide or viii) 0.1 μM pirenzepine. In addition, ACh induced a small inward current that was abolished in zero external Na. We conclude that ACh-1 pre-conditioning increases intracellular Na and thereby stimulates Ca^{2+} influx, possibly via Na/Ca exchange, to load SR Ca^{2+}. ACh-2 acts via M_1 muscarinic receptors to stimulate SR Ca^{2+} release and PKC via phospho-inositol hydrolysis. Potentiation of K current by ACh-2 is due to Ca^{2+}-dependent PKC activation of a glibenclamide-sensitive K current, presumably due to opening of ATP-sensitive K channels.

Th-Pos411

PURINERGIC STIMULATION ALTERS THE MEMBRANE PROPERTIES OF BROWN FAT CELLS. ((P.A. Pappone & S.C. Lee)) Section of Neurobiology, Physiology & Behavior, Univ. of Calif., Davis CA 95616.

We tested the effects of extracellular ATP on the membrane currents of patch clamped cultured neonatal rat brown adipocytes. ATP is present in the synaptic vesicles of the sympathetic neurons innervating brown fat and is released during normal activation of thermogenesis. In perforated-patch clamped cells, ATP (1-100 μM) 1) activated a voltage-independent, nonselective cation conductance, 2) turned on Ca-dependent K currents, 3) substantially reduced the amplitude of the voltage-gated K currents, $I_{K,V}$, and 4) increased the cell membrane capacitance, C_M, by as much as 25%. The ATP effects on $I_{K,V}$ and C_M cannot be due solely to changes in $[Ca^{2+}]_i$, since they are not seen with norepinephrine stimulation, which increases $[Ca^{2+}]_i$. Activation of the cation conductance and inhibition of $I_{K,V}$ also occurred with ATP exposure in whole-cell recordings with nucleotide-free pipet solutions, but no changes in C_M were seen under these conditions. Adenosine (10 or 100 μM) had no effect on the currents of ATP-responsive cells. These results suggest that brown fat cells have P_2 purinergic receptors that modulate their membrane properties.

Th-Pos412

THE FAST ADRENERGIC DEPOLARIZATION OF BROWN ADIPOCYTES IS MEDIATED BY A CA-ACTIVATED CHLORIDE CURRENT. ((P.A. Pappone & S.C. Lee)) Section of Neurobiology, Physiology & Behavior, Univ. of Calif., Davis, CA 95616.

The first measurable response to adrenergic stimulation of brown adipocytes is a rapid cell membrane depolarization. We used whole-cell and perforated-patch voltage clamp measurements in cultured neonatal rat brown fat cells to determine the ionic basis and mechanism of regulation of the fast depolarizing response. We find that the fast depolarization is due to a voltage-independent conductance increase to Cl^- ions, g_{Cl}. In perforated-patch clamped cells g_{Cl} can be activated by exposure to α-adrenergic agonists, Ca^{2+}-ionophore, or hypotonic solutions. In whole-cell experiments, sustained increases in g_{Cl} can be elicited with a nucleotide-free pipet solution that contains moderate concentrations of free Ca^{2+} (200-500 nM). However, similar pipet solutions with higher concentrations of free Ca^{2+} (≥ 1 μM) induce only transient activation of g_{Cl}. These results suggest that moderate increases in intracellular Ca^{2+} concentration activate g_{Cl}, while high concentrations of Ca^{2+}, still in the physiological range, inactivate the conductance.

Th-Pos413

PROTEIN KINASE A PHOSPHORYLATION AND G-PROTEIN REGULATION OF PURIFIED RENAL Na^+ CHANNELS IN PLANAR BILAYER MEMBRANES. ((I.I. Ismailov, J.H. McDuffie, and D.J. Benos)) Univ of Alabama at Birmingham, Birmingham, AL 35294.

Purified bovine renal epithelial Na^+ channels incorporated into planar lipid bilayer membranes were used to evaluate the biophysical consequences of its phosphorylation by protein kinase A (PKA). We also studied the effects of pertussis toxin-induced ADP-ribosylation on single-channel activity of non-phosphorylated and PKA-phosphorylated channels. PKA-induced phosphorylation increased channel activity; this increase was manifest both in terms of the number of multiple channel openings in the bilayer as well as by an increase in single channel open probability. The maximal increase in P_o occurred at ATP concentrations greater than 25 μM, and was dependent upon voltage; P_o increased to a maximum value of 0.10-0.15 at +40 mV, and to 0.35-0.45 at -40 mV. Thus, PKA conferred a voltage sensitivity to channel gating without affecting open channel conduction properties. PKA-phosphorylated Na^+ channels were inhibited by subsequent ADP-ribosylation with pertussis toxin (PTX). Addition of GTPγS reversed this inhibition. However, exposure of non-phosphorylated Na^+ channels to PTX increased channel open probability by a factor of 3 - 5. These results demonstrate that a cAMP-dependent pathway is an important regulatory element for amiloride-sensitive Na^+ channels, and that the effects of PTX-induced ADP-ribosylation of the channel-associated G_i protein on function depend upon the previous phosphorylation state of the protein. Supported by NIH Grant DK37206.

Th-Pos414

G PROTEIN STIMULATION ALTERS THE PROPERTIES OF CHROMAFFIN POTASSIUM CURRENTS. ((Shawna D. Cannon, Steven P. Wilson, and Kenneth B. Walsh)) University of South Carolina, School of Medicine, Columbia, SC 29208. (Spon. by S.R. Ikeda)

The whole-cell arrangement of the patch clamp technique was used to examine the effect of G proteins on K^+ channels in cultured bovine adrenal chromaffin cells. Internal dialysis of chromaffin cells with guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) or external application of AlF^-_4, to stimulate G proteins, resulted in a voltage-dependent increase in the amplitude of the outward K^+ currents. The half-maximal voltage required for activation of the currents was shifted by -16 mV in the presence of GTPγS. The augmentation in the K^+ currents was accompanied by the appearance of a fast component of current activation measured at potentials positive to 0 mV. The GTPγS-sensitive current could not be detected when internal K^+ was replaced with Cs^+ and was reversibly inhibited by tetraethylammonium (IC_{50} = 2 mM). In contrast, the scorpion venom charybdotoxin (50 nM) and the bee venom apamin (250 nM), only slightly reduced the K^+ currents during stimulation by GTPγS and did not alter the activation kinetics. In addition, the GTPγS-sensitive K^+ current could be activated in the absence of internal Ca^{2+}. Treatment of the chromaffin cells with fluoride decreased nicotine-evoked secretion of catecholamines in a concentration-dependent manner. Thus, bovine chromaffin cells contain a G protein-stimulated K^+ channel that may play a regulatory role in secretagogue-mediated exocytosis.

Th-Pos415

VOLTAGE-DEPENDENT GATING KINETICS OF THE α-TOXIN ION CHANNEL ARE MODULATED BY CHANGES IN IONIC STRENGTH ((John J. Kasianowicz)) NIST, Biotechnology Division, Gaithersburg, MD

We show that channels formed by *Staphylococcus aureus* α–toxin exhibit a voltage-dependent gating process that does not follow a classic equilibrium scheme with one open and one closed state. We also demonstrate that the voltage-dependent kinetics of channel closure are affected markedly by the concentration of monovalent cations in the bulk aqueous phase and that different cations affect the gating kinetics in different manners. For example, increasing the hydrogen ion activity increases the probability of channel closure at constant applied potential, an effect also elicited by di- and tri-valent cations [Menestrina. 1986. J. Membr. Biol. 90:177]. In contrast, we show here that increasing the concentration of monovalent cations other than H^+ decreases the probability of channel closure. We discuss the significance of these findings to possible mechanisms of voltage-dependent gating for the α–toxin channel.

Supported by a National Academy of Sciences/National Research Council Research Associateship.

Th-Pos416

ION PERMEABILITY PROPERTIES OF LIPID BILAYERS CONTAINING AMYLOID BETA-PEPTIDE AND ITS FRAGMENTS. ((Tajib Mirzabekov[1], Meng-chin Lin[1] and Bruce Kagan[1,2])) [1]Neuropsychiatric Institute of UCLA Medical School and [2]Brentwood Veterans Administration Medical Center, Los Angeles, CA 90024.

Alzheimer's disease is a neurodegenerative disorder characterized by formation of senile plaques in the brain. A major constituent of these plaques, amyloid beta-peptide (Aβ), has been implicated in neurotoxicity and the etiology of Alzheimer's disease. An 11-amino acid neurotoxic fragment of Aβ, Aβ25-35, can form voltage dependent ion permeable channels in planar lipid bilayers. Aβ25-35 were incorporated into proteoliposomes from which planar membranes were formed or was inserted into membranes directly from the aqueous phase. We observed the slow sigmoidal opening kinetics of these channels and the fast closing kinetics. Channels were turned off by Cu^{2+} and Cd^{2+}, but not by other divalent cations. Peptide aggregation may play a significant role in channel formation and gating. The planar membranes made from proteoliposomes with Aβ25-35 had ion channels with multiconductive levels and sharp voltage dependence. These membranes exhibited slight cation selectivity (Ca^{2+}:K^+: Na^+:Cl^- = 5.4:1.6:1.4:1). No channel formation was observed when membranes were made from proteoliposomes with Aβ1-40 or other fragments of peptide (Aβ25-28, Aβ25-31, Aβ28-35). "Aging" of Aβ1-40 and Aβ25-35 did not increase their channel forming activity.

Th-Pos417

ALZHEIMER'S DISEASE ß-AMYLOID AFFECTS INHERENT CHANNEL ACTIVITY OF PC12 CELLS. ((R.J. Pearce, R. Fukuyama, Z. Galdzicki, G. Ehrenstein and S.I. Rapoport.)) LNS, NIA and CNB, NINDS, NIH, Bethesda, MD 20892.

ß-Amyloid peptide (ß-AP) (1-40) is cytotoxic to neurons and has been reported to change the electrical conductance of biological membranes (Galdzicki et al., *Biophys. J. 64*: A101, 1993), and to form high conductance Ca-channels in black lipid membranes (BLM) (Arispe et al., *PNAS 90*:567-71, 1993). Using the single channel patch-clamp technique we examined the effect of ß-AP (4.6-46µM) on undifferentiated pheochromocytoma (PC12) cells. Cells were recorded from in the cell-attached mode after 1 day in culture with ß-AP included in the culture medium (serum-free DMEM), or by inclusion of the ß-AP in the patch pipette. The extracellular solution contained (mM): cholineCl 120, $CaCl_2$ 10, $MgCl_2$ 2, TEACl 10, HEPES 10, 4-aminopyridine 10, at pH 7.4. Single channel activity was observed in all cells exposed to ß-AP (n=13), but was very different from that reported in BLM by Arispe et al, (1993). PC12 cells which had not been exposed to ß-AP showed similar activity, though not so often (3/14 cells). Thus, rather than forming a *de novo* channel, ß-AP was up-regulating the activity of one already present in the membrane. A likely mechanism for this may be linked to the ability of ß-AP to increase Ca flux across the membrane of PC12 cells (Fukuyama et al., in preparation) and to activate a calcium-dependent channel. For cells not exposed to ß-AP in excised patches, it took several minutes for the activity to become apparent, suggesting that some other diffusible factor is involved in regulating this channel. The general characteristics of the channel in excised patches were the same as in cell-attached membrane patches. Preliminary data show a shift in the I-V relation with changes in Cl concentration, implicating chloride as the charge carrier for this conductance. These results indicate that ß-AP modulates channel activity, likely by augmenting Ca influx across the cell membrane.

Th-Pos418

NITRIC OXIDE INHIBITS A LOW-VOLTAGE-ACTIVATED POTASSIUM CONDUCTANCE IN MAMMALIAN TYPE I HAIR CELLS. ((W-.Y. Chen, R.A. Eatock)) Program in Neuroscience, University of Rochester, Rochester, NY 14642 and Dept. of Otolaryngology, Baylor College of Medicine, Houston, TX 77030.

Type I hair cells in mammalian vestibular organs are unusual in that they receive large cup-like (calyx) endings from primary afferent neurons. We have recorded voltage-dependent currents from type I hair cells isolated from rat semicircular canal organs. These cells have a large 4 AP-sensitive K^+ conductance ($I_{K,L}$) that activates above -90 mV, making their resting input resistances very low (15-50 MΩ). At a holding potential of -60 mV, the onset current evoked by voltage steps is largely attributable to $I_{K,L}$. This onset current was reduced when the nitric oxide (NO)-producing agents, sodium nitroprusside (SNP) and nitroglycerin (NTG), were added externally. 8-bromo-cGMP, the membrane-permeant analog of cGMP, also inhibited the onset current. Inhibition was measured as the percent decrease in onset current evoked by a step from -60 mV to -120 mV. The mean inhibitory effects produced by 1 mM doses were: SNP: 56±21% (s.d., n=14); NTG: 51±14% (n=4); 8-bromo-cGMP: 45±9% (n=4). Inhibition was also observed with 250 μM SNP (n=2) and 300 μM NTG (n=2). When superoxide dismutase and catalase (125 U/ml each) were added to SNP to prevent free radical formation, there was no change in the inhibitory effect (n=5). In the cell-attached patch configuration, with symmetrical 145 mM potassium gluconate solutions, a 29-pS channel was inhibited by 1 mM SNP (n=5) or 1 mM 8-bromo-cGMP (n=2). This channel may be the $I_{K,L}$ channel because it was blocked by 5 mM 4-AP and was active at -60 and -70 mV. The inhibition by SNP disappeared when cell-attached patches were excised, consistent with the involvement of soluble second messengers such as cGMP. These results raise the possibility that *in vivo*, $I_{K,L}$ can be turned off by retrograde messengers released by the calyx. This would augment receptor potentials by increasing the cells' input resistances.

Th-Pos419

INVOLVEMENT OF PHOSPHATASES IN RECEPTOR-DRIVEN POOL DEPLETION ACTIVATED Ca^{2+} INFLUX IN *XENOPUS* OOCYTES

((Anant B. Parekh and Heinrich Terlau)) Max-Planck Institut für experimentelle Medizin, Hermann-Rein Straße 3, D-37075 and Max-Planck Institut für biophysikalische Chemie, Am Faßberg, D-37077 Göttingen, Germany. (Spon. by D. Schild).

Depletion of IP_3 stores in Xenopus oocytes through activation of certain receptors (e.g. 5-HT) evokes Ca^{2+} entry. Some step in the cascade of events linking emptying of stores through exogenously expressed receptor stimulation to the subsequent Ca^{2+} influx is sensitive to protein phosphatase inhibitors, implicating a role for a phosphatase. We therefore have investigated the possible involvement of a phosphatase more directly. Bath application of a membrane-permeable chemical phosphatase, once pool-depletion Ca^{2+} influx had been initiated, rapidly inhibited this influx (as measured through the Ca^{2+}-dependent Cl^- channel activity). Elevation of Ca^{2+} through means independent of the IP_3 pathway were only weakly affected by this phosphatase, ruling out a major action of the phosphatase on the Cl^- channel or on cytosolic Ca^{2+} buffering. The results from injections of various protein phosphatases into the oocyte will be discussed. If Ca^{2+} influx following store depletion was blocked pharmacologically, removal of the blocker did not result in a larger Ca^{2+} entry despite the stores being now only partially refilled. Although other interpretations are possible, one is that the time-course of pool-depletion activated Ca^{2+} influx evoked by receptor stimulation may not be a simple function of the extent of store refilling.

Th-Pos420

TEMPERATURE-DEPENDENCE OF K_{CA} CHANNELS IN INSULIN-SECRETING CELLS.

((M.-L. Kohler and C. L. Stokes)) Department of Chemical Engineering, University of Houston, Houston, TX 77204-4792 (Spon. by A. Delcour)

Oscillations of membrane potential (V_m) in pancreatic β-cells, termed bursting, help regulate insulin secretion by controlling influx of Ca^{2+} through V_m-dependent Ca^{2+} channels. Electrical activity in *single* β-cells differs greatly from that in β-cells *within intact islets*, motivating our studies of ion channel behavior under the differing conditions of the experiments, particularly temperature (T; typically room T in single cell experiments, 37 °C in islets). The large conductance K_{Ca} channel in β-cells is rarely open at room T. To investigate further, we have characterized K_{Ca}-channel activity between 22 and 35 °C in the glucose-sensitive insulin-secreting cell line HIT-T15 under basal conditions (no glucose; nifedipine and glyburide to block Ca^{2+} and K_{ATP} channels, respectively) by patch clamp of cell-attached patches. Qualitatively, channel activity increases dramatically as T increases for a given V_m, with the greatest changes at the highest V_m. Quantitatively, as T increases from 22°C to 35°C, open probability increases, up to eight-fold at +70 mV. Mean closed time decreases as T increases (up to 85 percent reduction at +70 mV), while no significant change is observed in mean open time. Finally, current-V_m curves become steeper at higher T, revealing increased single-channel conductance (120 pS at 22 °C; 190 pS at 35 °C). These studies reveal that experimental T during patch clamp can dramatically affect the quantitative characteristics of channel activity in insulin-secreting cells. These studies of T-dependence of channel function in HIT-T15 and β-cells are continuing under stimulated conditions and for other channels.

Th-Pos421

THE Ca^{2+} RELEASE ACTIVATED CURRENT (I_{CRAC}) OF A MUCOSAL-TYPE MAST CELL IS SENSITIVE TO THE Cl^- CHANNEL BLOCKER 5-NITRO-2-(3-PHENYLPROPYLAMINO) BENZOIC ACID (NPPB).

((M. Reinsprecht, H. Schindler, C. Romanin)) Inst. f. Biophysics, Univ. of Linz, Austria.

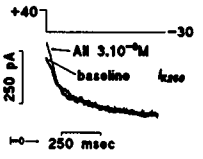
Immunologically-triggered secretion from mucosal-type mast cells (line RBL-2H3) is inhibited by the Cl^- channel blocker NPPB. In addition to its blocking activity on Cl^- channels of RBL-2H3 cells, here we provide evidence for an inhibition of I_{CRAC} by NPPB. The whole-cell configuration of the patch-clamp technique was employed to monitor I_{CRAC} activated by internal dialysis of RBL cells with 6.5 μM IP_3 in the presence of 10 mM EGTA. Potassium currents were largely eliminated by the use of Cs^+ in all solutions, and additionally 10 mM TEA^+ was included in internal solution. Activation of I_{CRAC} monitored by slow, repetitive (0.2 Hz) voltage ramps from -90 mV to +90 mV usually occurred within 30 s after obtaining whole-cell access. In standard extracellular solution supplemented by 10 mM Ca^{2+} netto I_{CRAC} was characterized by an inward current of about -20 pA at -75 mV and reversed at a potential around +60 mV. Extracellular application of 300 μM NPPB led to an about 90 % inhibition of I_{CRAC} determined at a potential of -75 mV. In experiments using 270 mM glucose instead of 140 NaCl in the extracellular solution characteristics if I_{CRAC} remained unchanged. Further, ionic substitution experiments providing Cl^- free condtions, and in addition leaving Ca^{2+} as the sole permeant cation did not markedly effect I_{CRAC} characteristics. Under each ionic condition I_{CRAC} was almost completely inhibited by 300 μM NPPB. These results suggest I_{CRAC} as a calcium entry pathway of RBL-2H3 cells and may explain the inhibitory effect of NPPB on secretion by its potency to inhibit I_{CRAC}. (Supported by Austrian Research Funds P9211)

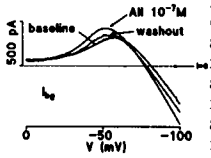
Th-Pos422

ANGIOTENSIN II MODULATES OUTWARD K^+ CURRENTS (I_K AND I_{K1}).

((P. Daleau & J. Turgeon)) Quebec Heart Institute, Ste-Foy, PQ, Canada.

Angiotensin II (AII)-activated receptor effects are mediated by intracellular 2nd messengers which alter ionic currents of heart cells. In this study, effects of AII (3.10^{-8}M) were assessed on voltage-dependent K^+ currents of guinea pig ventricular myocytes. Cells were superfused at 30°C with Cd^{2+}-containing solution to block I_{si}, held at -40mV to inactivate I_{Na} and currents measured in the whole cell configuration of the patch-clamp technique. I_K tail amplitudes were measured at -30mV after depolarization to various potentials (-20 to +50mV) for either 250msec (I_{K250}) or 5sec (I_{K5000}). I_{K1} was assessed by a ramp pulse from 0 to -100mV performed in 500msec. At +40mV test pulse, AII (n=8) increased I_{K250} by 14±8% but decreased I_K current measured during long depolarizing pulses (I_{K5000}) by 13±18% (mean±SD; both $P<.05$). Peak amplitude of background current was reversibly increased 20±17% by AII ($P<.05$). Increase in I_{K250} amplitude was associated with a more rapid decay in the early phase of deactivation of I_K suggesting an increase in the rapid component of I_K: I_{Kr}. Thus, results indicate that AII alters K^+ currents of ventricular cells and that modulation of AII levels may modify cardiac repolarization.





Th-Pos423

CHARACTERIZATION OF THE DIRECT EFFECTS OF ANDROGENIC/ANABOLIC STEROIDS ON LIGAND BINDING TO THE $GABA_A$ RECEPTOR.

((T.A.E. MASONIS and M.P. McCarthy)) CABM/RWJMS-UMDNJ, Piscataway, N.J., 08854.

Steroid hormones have profound effects on CNS function. Genomic and non-genomic mechanisms for modulation of $GABA_A$ receptor activity, by specific neuroactive steroids (NAS), have been documented. Due to the similarity between behavioral effects elicited by NAS, which modulate $GABA_A$ receptor activity, and the psychological changes observed in individuals abusing androgenic/anabolic steroids (AAS), we are investigating the effects of AAS on $GABA_A$ receptor function. Two commonly abused synthetic derivatives of testosterone, stanozolol and 17-methyltestosterone, were tested for their ability to modulate the binding of the $GABA_A$ receptor agonist [^{3}H]muscimol, to synaptoneurosomal membrane preparations from the cortex of 4-5 week old male and female Sprague Dawley rats. Interestingly, we have demonstrated enhanced binding of 25nM [^{3}H]muscimol (K_D=50mM) in the presence of nM to low μM concentrations of these AAS. The response exhibited variable concentration dependence and absolute magnitude in males and females. Multiple steroid binding sites are proposed, since multiphasic changes in [^{3}H]muscimol binding are observed as steroid concentration is increased. We are currently investigating the effects of these AAS on binding of other $GABA_A$ receptor ligands such as flunitrazepam and t-butylbicyclophosphorothionate (TBPS) in both males and females, as well as different brain regions, to further define their role in the modulation of $GABA_A$ receptor function.

Th-Pos424

KINETIC ANALYSIS OF ALCOHOL EFFECTS ON $GABA_A$ RECEPTOR-CHANNEL COMPLEX.

((Y. Kurata [a], W. Marszalec [a], J. Z. Yeh [a], B. J. Hamilton [b], D. B. Carter [b] and T. Narahashi [a])) [a] Dept. of Pharmacol., Northwestern Univ. Med. Sch., Chicago, IL 60611, [b] CNS Diseases Research, The Upjohn Company, Kalamazoo, MI 49001.

The effects of n-octanol on the kinetics of the $GABA_A$ receptor-channel were studied in human embryonic kidney cells expressing α_1, β_2 and γ_{2s} subunits. GABA evoked currents with an EC_{50} of 14.2 μM. n-Octanol augmented peak currents evoked by 3 μM GABA, decreased peak currents evoked by 300 μM GABA, suppressed the GABA response with pretreatment, accelerated the decay of GABA-induced currents, rapidly suppressed steady-state currents induced by 300 μM GABA, and itself produced small currents independent of GABA. These results were interpreted in terms of "mode transition" model. n-Octanol shifts the equilibrium between resting and activated modes to favor the activated mode, decreases the open probability of the activated mode, increases the rate of desensitization, and modifies receptor-channels rapidly, attaining equilibrium within seconds. We conclude that at least two alcohol-modified mode subsets are required to interpret the effects of n-octanol, suggesting that more than one molecular mechanism underlies the alcohol modulation of the $GABA_A$ receptor-channel.

Th-Pos425

THE APPARENT AFFINITY OF SUBSTANCE P FOR THE NEURONAL nAChR IS NOT DETERMINED BY A SINGLE DOMAIN OF THE β SUBUNIT. ((G.A. Stafford†, R.E. Oswald†, A. Figl‡, B.N. Cohen‡, H.A. Lester‡, and G.A. Weiland†)) †Dept. of Pharmacol., Cornell Univ., Ithaca, NY 14853 and ‡Div. of Biol., Caltech, Pasadena, CA 91125.

Our initial studies of substance P (SP) with various combinations of neuronal nAChR subunits expressed in *Xenopus* oocytes indicated that the type of β subunit present determines the apparent affinity of SP inhibition of ACh-induced current. The IC_{50} for SP is 67 ± 18 μM for α3β2 and 3.3 ± 0.43 μM for α3β4. By coexpressing chimeras of β2 and β4 subunits with α3, we have found that there is not a single discrete region of the β subunit that will reciprocally confer total β2- or β4-like SP effects. This is similar to what has been found for ACh (*Soc. Neurosci. Abstr.* 19:8, 1993). When the N-terminal portion of β2 is replaced with β4, the first 105 aa of β4 is enough to confer β4 ACh activity. In contrast, the IC_{50} for SP remains like β2. Extending β4 to aa 116 reduces the IC_{50} to ~20 μM, and the IC_{50} is unchanged when the β4 region is further extended to aa 254. Interestingly, a single point mutation that converts M2 of β4 to β2 (F255→V) also reduces the IC_{50} to ~20 μM. When the N-terminal portion of β4 is replaced with β2, the entire extracellular N-terminal domain of β2 is not able to confer β2 ACh activity. Extending β2 to aa 323, however, does (*i.e.*, 26 aa's into the putative intracellular loop between M3 and M4). Surprisingly, the IC_{50} for SP with this chimera increased 3.5-fold over β2 (240 μM), but the addition of another 98 aa's from β2 (*i.e.*, β2 to aa 431) conferred an IC_{50} like β2. Thus the apparent affinity of SP for the neuronal nAChR is determined by several domains of the β subunit, possibly including an indirect structural contribution by an intracellular domain.

Th-Pos426

REGULATION OF ATP-STIMULATED Cl^- CURRENT IN HT29-Cl.16E CELLS. ((X.W. Guo, R. Harvey, C. Laboisse and U. Hopfer)) Dept. of Physiology and Biophysics, CWRU, Cleveland, OH 44106 and INSERM U239, Faculte de Medecine, X.Bichat, Paris, France

The regulation of ATP-stimulated chloride current in goblet cell line HT29-Cl.16E was investigated by whole cell patch clamping and fluorescence imaging techniques. Under patch clamping, the basal current of the cells was 3 pA/pF and increased to 41.3±10.19 pA/pF after stimulation with 200 μM extracellular ATP (n=10, at +50 mV). The current-voltage relation was linear between -70 mV and +50 mV and the current lasted up to 1-2 min. The current was carried by chloride because the reversal potential was 0 mV under symmetric pipette and bath Cl^- solutions (145 mM) and shifted to +29 mV (close to the expected value) when Cl^- concentration was dropped to 35 mM in bath solution (n=6). The current was abolished when Cl^- was removed from solutions (n=3). 200 μM extracellular ATP also increased $[Ca^{2+}]_i$ of the cells from 70.73±3.3 nM to 459.4±49.5 nM (n=8). This $[Ca^{2+}]_i$ increase, which mainly came from intracellular pools, lasted about 3-4 min. The ED_{50} was ~1 μM ATP. 5 μm W13 (an inhibitor of Ca/Calmodulin) abolished the ATP-stimulated Cl- current by 77% (n=4) and abolished the ATP-stimulated $[Ca^{2+}]_i$ increase by 76% (n=21). However, TMB8 (an inhibitor of intracellular calcium release, 100 μM) decreased the ATP-stimulated intracellular calcium increase by 64% without any effect on ATP-stimulated Cl^- current. 100 nM ATP, which increased $[Ca^{2+}]_i$ to the same level as 200 μM ATP+100 μM TMB8 did, was not enough to activate the ATP sensitive Cl^- channels. These data and other suggest that ATP stimulates a process leading to activation of Cl conductance. This process is sensitive to W13 and depends on basal Ca but is independent of increase in intracellular calcium. Supported by NIH (DK-39658)

Th-Pos427

EXTERNAL ATP ACTIVATES A Ca^{2+}-DEPENDENT CATIONIC NONSELECTIVE CHANNEL IN OUTER HAIR CELLS OF THE GUINEA-PIG COCHLEA. ((T. Van den Abbeele, P. Tran Ba Huy and J. Teulon)), Lab. Otol. Exp., Fac. méd. Lariboisière and INSERM U323, Fac. méd. Necker, Paris, France.

The cell-attached and inside-out configurations of the patch-clamp technique were used to investigate the properties of a calcium-activated cationic channel (CAN) in the basolateral membrane of isolated outer hair cells (OHC). OHC of the apical turns from the guinea pig cochlea were mechanically dissociated and kept in a culture medium (L-15, Leibowitz) at room temperature. Conductive properties of the channel were studied in inside-out patches. Under symmetrical conditions (Ringer solution with NaCl 140 mM, KCl 4.8 mM, $MgCl_2$ 1.2 mM), the channel displayed a linear I/V relationship (unitary conductance 26.3 ± 0.3 pS n=15); when the bath NaCl was diluted to 42 mM, the reversal potential was shifted to + 27.4 mV ± 1.5 mV (n=9), indicating a high selectivity for cations (P_{Na}/P_{Cl} = 18); when replacing the bath NaCl with 140 mM KCl, the unitary conductance was not modified and the I/V relationship reversed near 0 mV (P_K/P_{Na} = 0.9). The open probability (Po) was dependent on the internal calcium level (threshold near 10^{-6} M). Adenine nucleotides inhibited the channel by decreasing Po without modification of the unitary conductance (AMP > ATP > ADP >> cAMP). Flufenamic acid and DCDPC, two blockers of the CAN channels, also decreased Po, without altering the unitary conductance. In cell-attached patches, the pipette containing Ringer solution or 145 mM Na-gluconate, the cationic channel could be activated *in situ* by superfusing the cell with ATP 10^{-5} M (n=11) or a calcium ionophore (ionomycin 10^{-6} M, n=7); in both cases, the mean delay of activation was lower than 2 min. This ATP-evoked activity could be reduced by superfusing the cell with ATP plus DCDPC, indicating that ATP activates cationic channels of the CAN-type. These results suggest that CAN-channels might participate in the control of membrane potential and modulate the excitability of OHC.

Th-Pos428

NEUROMODULATION OF CALCIUM CURRENTS IS ALTERED IN SUPERIOR CERVICAL GANGLION NEURONS OF SPONTANEOUSLY HYPERTENSIVE RATS. Chu Chen and Geoffrey G. Schofield. Department of Physiology, Tulane University. School of Medicine, New Orleans, LA 70112.

It is widely accepted that sympathetic nerve activity and Ca^{2+}-dependent release of norepinephrine (NE) are increased in spontaneously hypertensive rat (SHR). We previously reported that the biophysical properties and neuromodulation of Ca^{2+} currents are altered in renal efferent sympathetic neurons (RESN) of SHR. The purpose of the present study was to investigate if the altered neuromodulation of calcium channels is unique to RESN of SHR. Ca^{2+} currents of superior cervical ganglion (SCG) neurons from SHR and age matched normotensive WKY rats were characterized using the whole-cell patch-clamp technique. The inhibitory effect of NE (10 μM) on Ca^{2+} currents was attenuated in SCG neurons of SHR (40.1 ± 2.9%, n=13) as compared to that in WKY (56.2 ± 2.2%, n=11; $P < 0.01$). Similarly, 2-chloroadenosine (2-Cl-Ad, 10 μM)-induced inhibition of Ca^{2+} currents was also reduced in SHR (22.6 ± 4.6%, n=7) as compared to WKY (39.5 ± 3.1%, n=8; $P < 0.01$). Ca^{2+} current facilitation induced by large depolarizing conditioning pulses in the presence of NE or 2-Cl-Ad was less in neurons from SHR than in WKY. However, Ca^{2+} current facilitation was not significantly different between SHR and WKY after internal dialysis with a solution containing 200 μM GTP-γ-s. The results suggest that attenuated inhibitory effects of neurotransmitters on calcium currents may contribute to increased NE release per impulse from sympathetic neurons, and an altered neuromodulation of Ca^{2+} channel currents may be universal in sympathetic neurons of SHR.

Supported by PHS grant HL43656.

Th-Pos429

CHEMICAL MODIFICATIONS OF A VOLTAGE-DEPENDENT TRANSIENT POTASSIUM CURRENT IN RAT SUPRACHIASMATIC NUCLEUS NEURONS. ((R.-C. Huang and K.-W. Yau)) Howard Hughes Med. Inst. and Dept. of Neurosci., Johns Hopkins Univ. Sch. of Med., Baltimore, MD 21205.

The chemical modifiers N-bromoacetamide (NBA), N-bromosuccinimide (NBS), and chloramine-T (Ch-T) have been widely used to remove fast inactivation of Na and K channels, leading to the notion that the inactivation gates of both channels might be homologous in structure. We examined the effects of these reagents on a transient potassium current (I_A) in neurons dissociated from rat suprachiasmatic nucleus using the whole-cell patch clamp technique. This I_A is potentiated by micromolar concentrations of Zn^{2+}, which shifts its steady-state inactivation to more positive voltages (Huang et al., PNAS 1993, in press), possibly by binding to histidine residues (Huang and Yau, Soc. Neurosci. Abs. 19:292.13, 1993). External application of NBA irreversibly eliminated this I_A current, leaving the delayed rectifier. The I_A is mostly eliminated by 10 μM NBA in 5 minutes, and completely eliminated by 100 μM NBA in 1 minute. The NBA effects on the I_A current were mimicked by Ch-T but not by NBS, which had no effect on this current even at a concentration of 1 mM. The inhibitory effects of NBA and Ch-T were not accompanied by changes in the current kinetics, including the rate of inactivation. Thus, these observations contrasted with those reported in other cells, perhaps suggesting a heterogeneity of I_A channels.

Th-Pos430

TEDISAMIL BLOCK OF RAPIDLY AND SLOWLY INACTIVATING K^+ CURRENTS IN VISCERAL AFFERENT NEURONS . ((E.P. Christian and J.A. Togo)) Department of Pharmacology, ZENECA Pharmaceuticals Group, Wilmington, DE 19897.

We studied with whole cell recording techniques the effects of the Class III antiarrythmic agent, Tedisamil, on voltage-dependent rapidly (τ=10-50 ms; I_{A_F}), and slowly (τ_1=100-1200 ms, τ_2=1.1-7.8 sec; I_{A_S}) inactivating "A-like" K^+ currents in acutely dissociated neurons from the nodose ganglion of the adult guinea pig. Compatible with results reported previously from rat neonatal cultured nodose neurons (McFarlane and Cooper *J. Neurophysiol.* 66:1380-1391, 1991), these two currents were distinguishable by several parameters aside from inactivation rates, and both currents did not exist in every neuron. Activation and inactivation voltage-dependency curves of I_{A_F} were shifted negatively to those of I_{A_S} (Boltzmann $V_{½}$ coefficients: activation: I_{A_F}: -3 mV; I_{A_S}: +11 mV; inactivation: I_{A_F}: -54 mV; I_{A_S}: -28 mV). Additionally, I_{A_F} was insensitive to tetraethylammonium (1-20 mM), while peak I_{A_S} was blocked with an IC_{50} of ~1 mM. Tedisamil (0.1-100 μM) produced the following qualitatively similar concentration-dependent effects on both I_{A_F} and I_{A_S}: 1) an acceleration of the inactivation rate with little effect on the activation rate, and 2) a parallel hyperpolarizing shift of the inactivation voltage dependency curve with no effect on the activation voltage dependency. Tedisamil was ~5-fold more potent in exerting these effects on I_{A_S} vs. I_{A_F} (e.g., respective IC_{50}'s of 1.7 μM and 7.9 μM, for reducing the current integral due to the increased inactivation rate). In summary these results suggest a similarity in the inactivation gating mechanisms of the I_{A_F} and I_{A_S} in nodose neurons.

Th-Pos431

ACTIN FILAMENTS REGULATE ION CHANNELS IN IDENTIFIED NEURONS OF THE TIGER SALAMANDER RETINA. Greg Maguire* and Horacio Cantiello, *Sensory Sciences Center, The University of Texas, Houston, TX 77030 and The Renal Unit, MGH East, Harvard Medical School, Charlestown, MA 02129.

We are studying the effects of actin filaments on ion channel currents in identified retinal neurons in isolation and in a functional network. Whole cell (conventional and perforated patch) and single channel recordings were made from bipolar and amacrine cells in isolation and in the slice preparation. Neurons were identified from a battery of physiological and morphological criteria, including Lucifer yellow filling for morphological characterization in the slice. Initial studies indicate that voltage gated potassium channels are up-regulated by the depolymerizing agent cytocholasin D when it is added to the bathing medium (1-5 uM). This regulation is time dependent with the greatest enhancement of the current occurring 10-15 minutes post-treatment. When phalloidin was added to whole cell recording pipette and allowed to dialyze the neuron, bath applied cytocholasin D had no effect on the voltage gated potassium channels, i.e. the amplitude of the currents were no longer larger in the presence of cytocholasin D. We are now studying the effects of cytocholasin D on other channels, including chloride channels in amacrine cells, were the addition of cytocholasin D to the bathing medium results in an increased frequency of chloride channel openings, and the interaction between dopamine receptors and non-NMDA glutamate receptors.

Th-Pos432

Na^+-H^+ ANTIPORT VIEWED THROUGH HYDROGEN ION CURRENTS ((V.V. Cherny and T.E. DeCoursey)) Department of Physiology, Rush Medical Center, Chicago, IL 60612.

Voltage-activated H^+-selective currents were studied in rat alveolar epithelial cells and in human neutrophils using the whole-cell configuration of the patch-clamp technique. H^+ currents were depressed substantially, and the voltage-dependence of activation shifted to more positive potentials in Na^+, and to a smaller extent in Li^+, solutions compared with "inert" cations TMA^+, Cs^+ or NMG^+. We propose that the effects of Na^+ and Li^+ are due to their transport by the Na^+-H^+ antiporter, which is present in both cell types studied. Electrically silent H^+ efflux through the antiporter would increase pH_i and possibly decrease local pH_o, both of which modulate the g_H in a similar manner: reducing the H^+ currents at a given potential and shifting their voltage-dependence to more positive potentials. The reversal potential of the g_H was more positive in Na^+ than in inert ion solutions, consistent with depletion of protonated buffer in the cell by Na^+-H^+ exchange. 100 μM amiloride or 10 μM dimethylamiloride slightly inhibited H^+ currents in the presence of all cations studied except Li^+ and Na^+, in which they **increased** H^+ currents and shifted activation to more negative potentials. The inhibition by Na^+ was large when the proton gradient was outward (pH_o//pH_i 7//5.5), smaller with no gradient (pH 7//7), and absent when the proton gradient was inward (pH 6//7). Evidently, the Na^+-H^+ antiporter functions in dialyzed cells, producing pH changes detectable using the g_H as a physiological sensor. *Supported by a Grant-in-Aid from the American Heart Association with funds contributed by the A.H.A. of Metropolitan Chicago.*

Th-Pos433

REGULATION OF EPITHELIAL CELL JUNCTIONAL CONDUCTANCE BY CAMP. ((S. S. Garber[1] and M. Chanson[1,2])) [1]Dept. of Physiol., Medical College of PA, Philadelphia, PA and [2]Dept. of Med. Physiol. Univ. of Utrecht, Utrecht, Netherlands.

The human colonic cell line, T84, is often used as a model of epithelial cell regulation. Gap junctional proteins are expressed by T84 cells, as well as other epithelia. It has been suggested that cell-cell coupling may play a role in the control of fluid secretion. cAMP-dependent modulation of fluid secretion is well documented in T84 cells. A dual whole cell recording configuration was used to determine if cell-cell coupling in T84 cells, grown for 2-5 days on fibronectin coated glass cover-slips, was cAMP-dependent. In the absence of cAMP in the internal solution, only 6 out of 23 cell pairs exhibited cell-cell coupling with an average junctional conductance (g_j) of cells exhibiting coupling was 1.5 ± 0.8 nS. In contrast, cell-cell coupling was observed in 18/29 cells when non-hydrolysable analogs of cAMP (100 µM cpt-cAMP or the Sp diastereomer of cAMP-thioate, Sp-cAMP) were included in the pipette solution. Cells exhibiting coupling in the presence of cAMP analogs showed an average g_j = 5.4 ± 2.3 nS. This effect appears specifically cAMP-dependent as the Rp diastereomer of cAMP-thioate, a PKA antagonist, did not promote cell-cell coupling (n = 0/10). In cell pairs exhibiting coupling, the normalized junctional conductance ($g_j/g_{j(max)}$) was independent of the presence or absence of cAMP analogs and showed a shallow dependence on the transjunctional voltage (V_j). V_j was also independent of holding potential. These results suggest that cAMP-dependent regulation of junctional conductance in an epithelial syncitium can provide a mechanism for coordinating and regulating fluid secretion. Funded by NIDDK DK46672 and CFF I242.

Th-Pos434

EFFECTS OF HALOTHANE ON THE MUSCARINIC ACTIVATION OF G_K. ((J. Magyar, Y. Li and G. Szabo)) Dept. Mol. Physiol., Univ. Virginia, Charlottesville, VA22908.

Clinical concentrations of halothane (1.5%) can influence intracellular signal transduction, including G-protein mediated pathways. We have examined the effects of halothane on the muscarinic activation of potassium channels ($I_{K(ACh)}$) in atrial myocytes of the bullfrog (*Rana catesbeiana*). Since activation of $I_{K(ACh)}$ is coupled to receptor by the G protein G_K through a direct, membrane delimited process, $I_{K(ACh)}$ can be used as a rapid and specific indicator of G_K activation. We have characterized the time course of $I_{K(ACh)}$ development in response to rapid (~20 ms) applications of the agonist acetylcholine (ACh). A slightly altered K^+ concentration was used to validate the speed of the solution exchange. The time course of $I_{K(ACh)}$ response could be fit by $F=I_{max}\cdot(1-e^{-t})^4$ where I_{max} is the maximum amplitude of $I_{K(ACh)}$ and $f=m\cdot t-(1-e^{-m\cdot t})$ with t being the time relative to the onset of the agonist pulse and m a scale factor indicating the speed of the response. For 1 µM ACh we found m = 13±2.1 s^{-1} (n=7) for agonist alone and m = 18±2.5 s^{-1} (n=7) for agonist applied together with 2% halothane. In addition, I_{max} was decreased by 20% in the presence of halothane. These observations suggest that halothane significantly decreases the rate of G_K activation by agonist. The molecular basis of this effect remains to be elucidated. Supported by NIH Grant GM 47525

Th-Pos435

IONIC BASIS FOR THE ACTION OF COBRA SNAKE PHOSPHOLIPASE A_2 ON THE HEART: ELECTROPHYSIOLOGICAL EVIDENCE FOR ALTERED G PROTEIN FUNCTION THROUGH ENZYMATIC ACTIVITY. ((W. Wu, Y. Li, and G. Szabo)) Nat. Tsing Hua Univ. Inst. of Life Sciences, Hsinchu, Taiwan 30043 and Univ. of Virginia Sch. of Med., Charlottesville, VA 22908.

Snake venom phospholipase A_2 enzymes (PLA_2) affect a variety of tissues, typically producing neurotoxicity, cardiotoxicity and hemolysis. The molecular mechanisms of these pharmacologically significant effects are unclear. We have used the whole-cell configuration of the patch clamp technique to study the action of acidic PLA_2 isolated from the venom of the Taiwan cobra *Naja naja atrox* on the electrophysiological properties of bullfrog atrial myocytes. While at relatively low concentrations (up to 10 µM) extracellularly applied PLA_2 did not compromise membrane integrity, as indicated by the absence of any non-specific leak conductance, it had significant effects on endogenous ion channels. In particular, PLA_2 increased both basal and isoproterenol-induced Ca^{++} currents. This initial increase was followed by a slow decrease of both currents upon prolonged (>2 min) exposure. The initial enhancement of Ca^{++} currents was absent when chemically inactivated enzyme was used or G protein function was impaired by intracellularly applied GDPβS. In contrast, the slowly developing inhibition of the Ca^{++} currents was not affected by these maneuvers but it could be mimicked by extracellular application of arachidonic or oleic acid. Since PLA_2 also impeded muscarinic cholinergic activation of the K^+ channel $I_{K(ACh)}$, its action may be rationalized as a rapid inhibition of basal G_i activity that leads to a reduced constitutive inhibition of adenylyl cyclase and thereby to enhaced Ca^{++} channel activation.

Supported by NIH Grant HL37127

Th-Pos436

THE PACEMAKER CURRENT I_F IN NEONATAL AND ADULT MAMMALIAN VENTRICLE CELLS. ((R.B. Robinson, H. Yu, F. Chang, V. Kuznetsov, and I.S. Cohen)) Columbia University and SUNY Stony Brook, New York.

The pacemaker current i_f contributes to spontaneous activity in the mammalian sinus node and Purkinje fibers, but has been presumed absent in nonautomatic tissue such as ventricle. The recent observation of i_f at very negative potentials in adult dog and guinea pig ventricle cells raises questions about the function of the current in these tissues. We used neonatal and adult rat ventricle cells to test the hypothesis that i_f is present at relatively positive potentials in young ventricle tissue, and shifts to more negative voltages with age. After enzymatic dispersion and culture, 9 neonatal cells studied all exhibited a time-dependent inward current on hyperpolarization; 4 mM Cs was tested in 8 of the cells and reversibly inhibited the current in all of them. The activation threshold of the current in these cells was -70±2 mV (x±SEM). The relatively positive voltage threshold suggests that i_f may contribute to the spontaneous activity of these cells in culture. This was confirmed by experiments where 1 mM Cs, a concentration that should be selective for i_f, slowed automaticity by 33% (from 69±5 to 49±12 beats/min; n=6). In separate experiments 1 mM Cs reduced i_f at -75 mV by 85±7% (n=3). All 12 adult rat ventricle myocytes studied exhibited an i_f-like current; Cs was tested in 9 of the 12 cells, and was effective in inhibiting the current in all of them. However, relative to the neonatal cells, the activation threshold was shifted over 40 mV negative in adult cells, to -113±6 mV. The difference in activation threshold did not result from the culture of the neonatal cells, since neonatal cells which were dissociated and acutely studied like the adult preparation exhibited a relatively positive threshold (-72±2, n=6; Cs blocked in each cell). These results suggest that developmental determination of pacing and nonpacing tissue, and the normal slowing of spontaneous heart rate with maturation, may in part be controlled by regulating the position of the current i_f on the voltage axis.

Th-Pos437

HALOTHANE INHIBITS AGONIST-EVOKED Ca^{2+} INFLUX IN BOVINE AORTIC ENDOTHELIAL CELLS. ((C. Simoneau, G. Blaise* and R. Sauvé)) Membrane Transport Research Group, Depts. Physiology and Anaesthesia*, Univ. Montréal, Montréal, Canada H3C 3J7.

Volatile anaesthetics such as halothane and isoflurane have been reported to affect vascular tone. In many cases, this effect was linked to the presence of an intact endothelium. Because the cardiovascular action of endothelial cells involves the production and secretion of vasoactive agents such as EDRF via a Ca^{2+}-dependent process, a fura-2 study was undertaken to determine the effect of the volatile anaesthetic halothane on the Ca^{2+} response of bovine aortic endothelial cells (BAEC) to bradykinin (BK) stimulation. Our results essentially indicate **1)** that halothane (1-5%) does no cause *per se* a release of Ca^{2+} from internal Ca^{2+} pools but reversibly reduces the cytosolic Ca^{2+} increase evoked by BK; **2)** that this inhibitory effect is not due to an action on the BK-related $InsP_3$ production; **3)** that halothane does not affect the uptake of Ca^{2+} in $InsP_3$-sensitive Ca^{2+} pools and finally **4)** that the influx of Ca^{2+} in BK-stimulated cells is reduced in the presence of halothane. It is concluded that the inhibitory action of halothane results either from a direct action on the structure responsible for the receptor-evoked Ca^{2+} entry in these cells or through an effect on one or several intracellular second messengers related to Ca^{2+} pool depletion. Work supported by Heart and Stroke Foundation of Québec.

Th-Pos438

ACTIVATION OF ADENOSINE-1 RECEPTORS INCREASES STEADY-STATE OUTWARD CURRENT IN METABOLICALLY INTACT CORONARY SMOOTH MUSCLE CELLS. ((Eric P. Kuivinen and Richard E. White)) Dept. Physiology & Biophysics, Wright State University School of Medicine, Dayton, OH 45435.

Adenosine induces a potent vasodilation of coronary arteries which is associated with changes in membrane potential; however, neither the specific ion channels modulated by adenosine nor the signaling mechanisms have been identified. We have investigated the effects of adenosine on single metabolically intact smooth muscle cells isolated from porcine coronary arteries. Cells were bathed in physiological saline and voltage-clamped at a holding potential of -60 mV through membrane patches permeabilized with nystatin. In the presence of 10 μM R-phenylisopropyl-adenosine (RPIA), a selective A-1 adenosine receptor agonist, the steady-state outward current was increased by 85.8 ± 26% at +40 mV. This increase in current was not affected by 10 μM glibenclamide or 5 mM 4-aminopyridine, but was inhibited >70% by 1 mM tetraethylammonium (TEA). 1 mM 8br-cGMP had a similar effect, increasing outward currents by 60.0 ± 14% at +40 mV. In cell-attached patches, R-PIA or 8br-cGMP increased the opening frequency of a TEA-sensitive, glibenclamide- and 4-aminopyridine-insensitive channel with an extrapolated reversal potential near E_K. These findings suggest that activation of A-1 receptors stimulates the activity of the large conductance, calcium- and voltage-activated potassium channel in coronary smooth muscle cells, possibly by activating the cGMP transduction pathway. *(supported by the National Heart Foundation/AHAF, AHA/Ohio Affiliate, and WSU241525).*

Th-Pos439

FAILURE TO DETECT A PROTEIN KINASE A-REGULATED CHLORIDE CURRENT IN HUMAN ATRIAL MYOCYTES. ((S. Sorota§, E.A. Rose* and M.C. Oz*)) Dept of Phamacology§ and Dept. of Surgery*, Columbia University, New York, NY 10032

Pieces of right atrium were obtained from patients undergoing coronary artery bypass surgery. Single human atrial cells were isolated by digestion with collagenase and protease. Whole cell patch clamp was used. Potassium-containing solutions were used so that action potentials could be measured before proceeding with voltage clamp studies. Steady-state membrane currents were recorded in response to -17 mV/sec voltage ramps. Isoproterenol (1 μM, with 100 μM GTP in the pipette solution) had no effect on steady state membrane currents of two atrial cells. Forskolin (10 μM) had no effect on steady state membrane currents in four atrial cells from two different patients. As a positive control, net inward currents during voltage clamp steps from -50 to -5 mV were also measured. In all cells, isoproterenol or forskolin induced a large increase in net inward current during voltage clamp steps, suggesting that PKA-dependent regulation of the L-type calcium current was intact. Although mRNA for a CFTR-like chloride channel has been detected by others in human atrium (Levesque et al Circ.Res. 71:1002-1007, 1992) the present results suggest that functional channels do not accumulate in human atrial myocytes. We cannot exclude however, the possibility that the channel proteins may have been damaged during cell isolation. (S.S. is supported by an Investigatorship and Grant-in-Aid from NYC Affiliate of AHA)

Th-Pos440

OXY-RADICAL STRESS ACTIVATES NON-SELECTIVE CATION CURRENT IN GUINEA-PIG VENTRICULAR MYOCYTES: ROLE OF SULFHYDRYL GROUPS. ((R.I. Jabr and W.C. Cole)) Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1.

Oxygen-derived free radicals (O-R) are reported to induce alterations in cardiac electrical activity including sustained depolarization. However, the ionic current(s) involved and mechanism(s) by which it is modified by O-R stress are unclear. In this study, we employed the whole-cell variant of the patch-clamp technique to determine the steady-state ionic current(s) mediating O-R induced depolarization in guinea-pig ventricular myocytes during extracellular application of the O-R generating system, dihydroxyfumaric acid (DHF; 3mM) and $FeCl_3$:ADP (0.05:0.5mM). Myocytes superfused with the O-R and dialyzed with pipette solution containing EGTA (0.1mM) show sustained depolarization to between -35 and -20 mV due to a positive shift in reversal potential (E_r) of the quasi steady-state I-V relation from -78.3 ± 1.4 to -27.6 ± 4.8 mV ($p < 0.001$; n=6). E_r of the difference current was -18.3 ± 2.1 mV, consistent with the activation of a non-selective cation conductance (I_{NSC}) as previously shown for intracellular O-R stress (Jabr & Cole, Circ Res 1993;72:1229). I_{NSC} (recorded selectively with 10μM TTX, 10μM nicardipine and replacement of K^+ with Cs^+) was still observed during O-R stress in myocytes; 1) pretreated with 10μM ryanodine, or 2) dialyzed with BAPTA (5mM) and superfused with Ca^{2+} and Na^+ free solution. This suggests that I_{NSC} was not activated by a rise in $[Ca^{2+}]_i$ due to O-R stress. However, I_{NSC} activated by O-R was inhibited by the sulfhydryl (SH) group reducing agent, dithiothreitol (DTT; 0.5-1mM). Moreover, the SH group oxidizing agent, diamide (0.5-1mM), was found to activate I_{NSC}, leading to a positive shift in E_r of steady-state current from -60.3 ± 4.1 to -15.4 ± 1.9 mV ($p < 0.001$; n=5) which could be reversed with DTT (0.5-1mM). These data suggest that extracellular O-R stress activates I_{NSC} through oxidative modification of SH groups in the channel proteins. Supported by MRC. RJ is a CHSF trainee.

Th-Pos441

A SINGLE TYPE OF CHLORIDE CURRENT (I_{Cl}) INDUCED BY ACTIVATION OF PROTEIN KINASES A AND C IN FELINE VENTRICULAR MYOCYTES ((Ke Zhang and R. E. Ten Eick)) Northwestern University, Chicago, IL 60611

We have previously reported circumstantial evidence suggesting that the cAMP-dependent, protein kinase A (PK-A) mediated chloride current (I_{Cl}) and the phorbol 12-myristate 13-acetate (PMA)-induced, protein kinase C (PK-C) mediated I_{Cl} in cat ventricular myocytes are conducted through the same set of Cl^- channels. If this hypothesis is true, maximal activation of I_{Cl} by PMA should prevent a subsequently applied maximal concentration of isoproterenol (ISO) or forskolin (FSK) from activating any additional I_{Cl}. However, if separate channel types are involved, the subsequently applied ISO or FSK should result in more I_{Cl} than produced by the PMA alone. The results indicate that indeed a maximal concentration of PMA prevented ISO or FSK from activating any further I_{Cl}. 4α- and 4ß-phorbol could not mimic this effect. The possibility that PMA may have inhibited the cAMP dependent pathway in addition to having activated all PK-C dependent Cl^- channels was investigated. When the effect of PMA on I_{Cl} was prevented by inhibiting PK-C with either staurosporine or calphostin-C, PMA did not alter the size of the I_{Cl} activated by ISO or FSK. These results strongly suggest that both PMA acting through PK-C and ISO acting through PK-A activate the same set of Cl^- channels. The several control experiments appear to make other possible explanations for the results highly unlikely.

Th-Pos442

TETRAETHYLAMMONIUM (TEA) BLOCKS MUSCARINIC REGULATION OF THE ISOPROTERENOL ACTIVATED CHLORIDE CURRENT IN CARDIAC VENTRICULAR MYOCYTES ((S.I. Zakharov, R.A. Wagner, and R.D. Harvey)) Dept. of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106

It has previously been shown that tetramethylammonium (TMA) antagonizes β-adrenergic activation of the protein kinase A (PKA) regulated Cl^- current in guinea-pig ventricular myocytes through muscarinic receptor stimulation (Zakharov et al., Am. J. Physiol. 264:C1625-C1630, 1993). Therefore, we tested the possibility that another quaternary alkylammonium compound, tetraethylammonium (TEA), may also affect muscarinic regulation of ion channels in cardiac muscle. Macroscopic currents were recorded using the whole cell configuration of the patch clamp technique. Following activation of the PKA regulated Cl^- current with isoproterenol (ISO), concurrent exposure to TEA (10 mM) had no effect. However, the muscarinic receptor agonist acetylcholine (ACh) reduced the ISO-activated current in a concentration dependent manner. The dose-response relationship was well described by a logistic equation, where the concentration of ACh that resulted in half-maximal inhibition of the current activated by 1 μM ISO (EC_{50}) was 30 nM, and the maximum inhibition was 76%. Atropine blocked the response to ACh in a competitive manner, causing a parallel shift of the ACh dose-response relationship to the right, without affecting the magnitude of the response to saturating concentrations of ACh. Similar to the effect of atropine, TEA also blocked the response to ACh, causing a parallel, concentration-dependent shift of the ACh dose-response relationship to the right, without affecting the maximum response to ACh. The equilibrium constant (K_a) for TEA dissociation from the muscarinic receptor was 41 μM, as determined by Schild analysis. These results indicate that TEA acts as a competitive antagonist of the muscarinic receptor in cardiac myocytes.

Th-Pos443

RECOMBINANT Gβγ ACTIVATES THE INWARDLY-RECTIFYING ATRIAL POTASSIUM CHANNEL I_{GIRK1}. (K. Wickman*, J.A. Iniguez-Lluhi*, P.A. Davenport*, R. Taussig*, G. Krapivinsky*, A.G. Gilman*, D.E. Clapham*) Departments of Pharmacology, *Mayo Foundation, Rochester MN 55905, and *University of Texas Southwestern Medical Center, Dallas TX 75235

One mechanism for controlling heart rate involves the release of acetylcholine (ACh) from the vagus nerve to pacemaker cells of the sino- and atrioventricular nodes and to the atria. ACh binds to muscarinic receptors resulting in the activation of inwardly-rectifying potassium channels (I_{GIRK1}, previously $I_{K,ACh}$) via pertussis toxin-sensitive G proteins. We addressed the issue of I_{GIRK1} regulation using both purified native and recombinant G protein subunits. Six recombinant Gβγ combinations activated I_{GIRK1} in inside-out patches from atrial myoctes with half-maximal activation concentrations ranging between 3-30nM. In addition, both native and recombinant Gα-GDP reversed I_{GIRK1} activity elicited by Gβγ and GTPγS in a dose-dependent manner. These results indicate that Gβγ is a specific activator of this channel, and suggest that Gβγ is the physiological mediator of I_{GIRK1} activation. Activation of I_{GIRK1} by recombinant Gα-GTPγS was observed, but evidence suggests that this was due to release of GTPγS from the protein. We conclude that Gβγ is a primary regulator of I_{GIRK1} activity.

Th-Pos444

ALPHA$_1$-ADRENERGIC MODULATION OF WHOLE CELL POTASSIUM CURRENTS IN RAT AND HUMAN ATRIAL MYOCYTES. ((M. Lamorgese and D.R. Van Wagoner)), The Cleveland Clinic Foundation, Cleveland, OH 44195.

Recent studies in several animal species have shown that α_1-adrenergic agonists can suppress outward K currents in cardiac muscle. In view of the high α_1-adrenergic receptor density in rat heart, we have begun to examine this effect in isolated adult rat atrial myocytes. The transduction pathways linking receptor activation to the suppression of K^+ currents are still poorly characterized. The nystatin perforated patch technique was used to measure stable whole cell K^+ currents. Phenylephrine (PE, 10 μM, with 1 μM propranolol) was used as the α_1-adrenergic agonist. Unexpectedly, analysis of the difference currents showed that PE had no significant affect the amplitude of the transient outward K current (I_{TO}), but rather, caused a rapid suppression of the delayed rectifier K^+ current (I_K). PE caused a 29±6% decrease in I_K compared to control, $p<0.01$, $n=17$). Similar results have been observed in 4/4 human atrial myocytes dissociated from atrial appendages removed from patients during routine cardiac surgery. Suppression of I_K increases action potential duration and enhances calcium influx. These results suggest that suppression of I_K may contribute to both the positive inotropic and pro-arrhythmic effects of catecholamines. We are currently exploring the mechanisms transducing this effect.

Th-Pos445

ELONGATION OF ACTION POTENTIAL DURATION IN RAT ATRIAL MYOCYTES BY RELAXIN. ((E. S. Piedras-Rentería, O. D. Sherwood and P. M. Best.)) Dept. Physiology & Biophysics, University of Illinois, Urbana, IL 61801.

Relaxin, a hormone best known for its role in pregnancy, has chronotropic and inotropic effects in the rat heart, where specific relaxin receptors are found only in the atria. We studied the effect of rat relaxin on the action potential of enzymatically dispersed atrial cells from 4.5-8 week-old female and male Fischer rats. The bath solution composition was (mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES; the pipette solution contained: 100 KCl, 10 K_2EGTA, 8 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 0.2 Na_2GTP, 7 Na_2ATP, 15 phosphocreatine, 40 μg/ml creatine phosphokinase. Action potentials were elicited by injection of 2 ms pulses of 1.8 nA from holding potentials of -94.9 ±2.9 mV. Addition of 50, 100 or 200 ng/ml of rat relaxin increased the action potential duration (APD_{90} = action potential duration at 90% of repolarization) by 26 ±1.5% ($n=3$)*, 46 ±3% ($n=9$)* and 56.9 ± 20% ($n=3$), respectively (*$p\leq0.05$); this increase was also seen in the presence of 1 μM propranolol. Inclusion of 2 μM of a protein kinase A inhibitor (5-24 amide) in the pipette prevented the effect of 100 ng/ml of relaxin. Our results suggest that the relaxin effect on isolated atrial cells is mediated by relaxin receptors and coupled to protein kinase A. The elongation of the action potential by relaxin could account for the inotropic effect of the hormone observed in the cardiac atrium. Voltage clamp experiments showed no effect of relaxin on L-type calcium currents; effects on other ionic currents are being investigated.
Supported by AHA, Illinois (P.M.B. and E.S.P-R.) and N.I.H. (O.D.S.).

LIGAND-GATED CHANNELS

Th-Pos446

CLONING, FUNCTIONAL EXPRESSION, AND PHARMACOLOGICAL CHARACTERIZATION OF HUMAN NMDAR1/NMDAR2 HETEROMERIC RECEPTORS ((S.D. Hess, C.-C. Lu, L. Chavez-Noriega, A. Urrutia, J. Crona, L. Daggett, R. Heckendorn, C. Angst, H. Allgeier, E. Johnson, M. M. Harpold, G. Velicelebi)) SIBIA, Inc. 505 Coast Blvd. S., La Jolla, CA 92037 and CIBA-Geigy, CH 4002 Basel, Switzerland.

Overlapping cDNA clones that encode human NMDAR1A, NMDAR2A, and NMDAR2B subunits were isolated. *In vitro* transcripts for these subunits were injected into *Xenopus* oocytes and assayed by two-electrode voltage clamp. Coexpression of NMDAR2A or 2B with NMDAR1A markedly potentiated inward currents elicited by glycine and NMDA or glutamate as compared to NMDAR1A homomeric receptors. There were no significant differences for NMDAR1A/R2A and R1A/R2B heteromeric receptors with respect to activation by NMDA/glycine and glutamate/glycine. For both receptors, glutamate was more potent than NMDA. The heteromeric receptors were also tested for their sensitivity to NMDA antagonists. The rank order of potency for the antagonists was CGP 40116 > CPP > CGS 19755 ≈ CGP 43487 for NMDAR1A/2A and CGP 40116 > CGS 19755 > CPP > CGP 43487 for NMDAR1A/R2B. There were no significant differences in the sensitivities of the human NMDAR1A/R2A and R1A/R2B receptors to CGS 19755 or CGP 40116. However, the NMDAR1A/R2A receptor was 8 fold more sensitive to CPP and 3 fold more sensitive to CGP 43487 than the R1A/R2B receptor.

Th-Pos447

CLONING AND FUNCTIONAL CHARACTERIZATION OF THREE SPLICE VARIANTS OF THE HUMAN NMDAR1 RECEPTOR. ((L. Daggett, S.D. Hess, C. Liaw, A. Urrutia, E. Johnson, S.B. Ellis, M.M. Harpold, and G. Velicelebi)) SIBIA, Inc., 505 Coast Blvd. S., La Jolla, CA 92037

Oligonucleotide probes based on the rat NMDAR1 sequence were used to screen a human hippocampal library and isolate overlapping cDNAs corresponding to alternatively spliced transcripts encoding human NMDAR1 subunits. The splice variant NMDAR1AΔ363 has a 363-nt deletion starting at position 2700, 3' to the fourth putative transmembrane region, and NMDAR1AI63 has a 63-nt insert between nucleotides 570 and 571, 5' to the first putative transmembrane region. *In vitro* transcripts for these receptors were injected into *Xenopus* oocytes and assayed by two-electrode voltage-clamp. At -80 mV, application of 10 μM glutamate and 10 μM glycine elicited inward currents of 15-21 nA in cells injected with any of the three transcripts. The EC_{50} values determined from 3-5 cells with glutamate for NMDAR1A, NMDAR1AΔ363 and NMDAR1AI63 were 0.4, 0.5 and 0.6 μM, respectively, and 6.3, 11.9 and 10.9 μM for NMDA, respectively. These responses were blocked by 1 μM MK-801 or 100 μM Mg^{2+}. Our data show that these splice variants encode functional homomeric receptors.

Th-Pos448

RECOMBINANT GLUTAMATE-RECEPTOR ION CHANNELS FORMED FROM GLUR6 SUBUNITS. ((John Marshall and James R. Howe)) Dept. of Pharmacology, Yale University School of Medicine, New Haven , CT 06520.

Patch-clamp methods were used to study glutamate-receptor ion channels of the kainate subtype expressed in adenovirus-transformed human embryonic kidney cells (HEK 293). Kainate evoked large sustained currents in about 30% of the cells tested 3 to 4 days after they were transiently transfected with cDNA encoding the GluR6 subunit. (Cells were treated with concanavalin A (25 μM) to remove rapid desensitization.) The fits to concentration-response data for kainate-evoked currents gave mean EC_{50} and n_H values of 0.34 μM and 1.46, respectively (n = 5). The corresponding values obtained for kainate in the presence of CNQX (20 μM), a competitive antagonist of non-NMDA receptors, were 2.7 μM and 1.02 (n = 2). At -80 mV, kainate typically evoked maximal inward currents of 300 to 500 pA which were not associated with a detectable increase in current noise. In two cells where the recording conditions were especially good, power spectra of kainate-evoked noise contained two Lorentzian components and gave single-channel conductance (γ_{noise}) estimates of 72 and 140 fS, respectively. In several outside-out patches from cells that responded to kainate we were unable to detect discrete single-channel currents. Instead, at -80 mV kainate produced macroscopic-like inward currents of 2 to 6 pA during which there was only a small increase in current noise. In our best patch, analysis of this noise gave power spectra well fitted by two Lorentzians with respective half-power (f_c) frequencies of about 20 and 280 Hz. Values for γ_{noise} ranged from 210 to 280 fS (n = 12). Values for f_c and γ_{noise} did not depend on membrane potential (-80 to +40 mV) or on the kainate concentration (0.1 to 10 μM), but the relative amplitude of the low-frequency component (f_c = 20 Hz) increased with agonist concentration.

Th-Pos449

POTENTIATION OF 5-HT3 RECEPTOR FUNCTION BY ALCOHOLS
((Q. Zhou and D. Lovinger)) Dept. Mol. Physiol./Biophys., Vanderbilt , Nashville, TN 37232

We have previously reported that ethanol (EtOH), ether, trichloroethanol (TCEt), and dichloroethanol (DCEt) potentiate ion current mediated by 5-HT3 receptors. These drugs appear to act on different sites on the receptor. Butanol (ButOH) and isopentanol (IsopOH), and the TCEt analogoue, trifluoroethanol (TFEt), also increase the amplitude of current induced by 1μM 5-HT in NCB-20 neuroblastoma cells. IsopOH is the most potent one among them with an EC50=1.15 mM. But its maximum efficacy (71.5%), is only slightly greater than that of EtOH (54.1%). TFEt exhibits relatively low potency (EC50=27.3mM) similar to that of EtOH (EC50=30.5mM) but TFEt exhibits a maximum efficacy (204.5%) which is much greater than EtOH. ButOH exhibits intermediate potency (7.93mM) and efficacy (123.7%). Coapplication of maximally effective concentrations of both ButOH (20mM) and TCEt (20 mM) induces potentiation which lies midway between the effect of either drug alone. At low concentrations of TCEt (1mM) and ButOH (5mM), coapplication of both drugs produces a larger potentiation than either drug alone. Application of EtOH does not alter responses to coapplied ButOH or TCEt. TFEt does not alter the effect of TCEt on the 5-HT3 receptor either. Applying 5 mM TFEt with 1 mM TCEt gives the same amount of potentiation as TCEt alone. Similar results have been obtained using high concentration of TCEt and TFEt.

Potentiation(%)

20mM Butanol	106±14.4	100mM Ethanol	25±4.75	100mM Ethanol	51±29	100mM TFEt	225.6±93.1
20mM TCEt	320±45	20mM Butanol	157±26.5	25mM TCEt	462±233	25mM TCEt	274.5±156.4
Butanol+TCEt	230±53	Ethanol+Butanol	164±40	Ethanol+TCEt	441±228	TFEt+TCEt	216.4±121.1
5mM Butanol	15.4±4.2	25mM Ethanol	6.8±3.3	25mM Ethanol	5.5±3.9	5mM TFEt	20±23.9
1mM TCEt	50.3±7.4	5mM Butanol	72±13.3	1mM TCEt	63.8±30	1mM TCEt	80.3±58.3
Butanol+TCEt	65±9.8	Ethanol+Butanol	72.4±8.5	Ethanol+TCEt	54±30	TFEt+TCEt	71.5±42.8

Each drug tested has a unique pharmacological profile and interaction with other compounds. While drug hydrophobicity was closely related to potency, it was unrelated to maximum efficacy. This suggests that bulk lipid fluidity changes may not account for effects on this receptor. Supported by the ABMRF and NIAAA.

Th-Pos450

EFFECTS OF PICROTOXIN AND *tert*-BUTYLBICYCLOPHOSPHOROTHIONATE ON $GABA_A$-ACTIVATED CURRENT IN RECOMBINANT RAT $GABA_A$ RECEPTORS. ((G.H. Dillon, B.J. Hamilton, D.B. Carter and W.B. Im)) Upjohn Labs, Kalamazoo, MI 49001. (Spon. by D.E. Epps)

Picrotoxin (Px) and *tert*-butylbicyclophosphorothionate (TBPS) are well-known inhibitors of neuronal $GABA_A$-activated Cl^- current, and share the same binding site on the $GABA_A$ receptor/channel complex, presumably near the mouth of the Cl^- channel. In this study, we attempted to characterize their interaction with the $\alpha1\beta2\gamma2$ subtype of recombinant $GABA_A$ receptor expressed in human embryonic kidney cells, using the whole-cell configuration of the patch clamp technique. Inward currents induced by 5 μM GABA at a holding potential of -60 mV (symmetrical Cl^- gradient) decayed very slowly (tau > 200 s from one exponential function) during 15 s application of GABA. Both Px and TBPS increased the rate of current decay and decreased peak current amplitude in a concentration-dependent manner. The K_D for Px in the absence of GABA, determined from its effect on initial peak current, was 11.4 μM, while the K_D in the presence of GABA, estimated from the drug concentration-dependent changes in tau, was 443 nM, indicating the affinity for Px is increased when $GABA_A$-activated channels are open. Corresponding values for TBPS were 2.2 μM in the absence of GABA, and 70 nM in the presence of GABA. Recovery from both compounds followed a monoexponential time course. Preincubation with 200 nM GABA did not alter the effects of Px or TBPS on peak current amplitude or tau for current decay. These results suggest that Px and TBPS have greater affinity for multi-liganded (both high and low affinity GABA binding sites occupied) $GABA_A$ receptors than non- or mono-liganded (only high affinity binding site occupied) receptors.

Th-Pos451

KINETICS OF $GABA_A$ RECEPTOR CHANNELS STUDIED WITH RAPID APPLICATION TECHNIQUES. ((M.V. Jones and G.L. Westbrook)) The Vollum Institute for Advanced Biomedical Research, Portland, OR 97201 (Spon. G.L. Westbrook)

Neurotransmitter is present for only a few milliseconds at central excitatory synapses, reaching a peak concentration in the millimolar range.[1] Thus channel kinetics shape the resulting excitatory synaptic current. However, there is limited information about the transmitter timecourse and synaptic channel kinetics at central inhibitory synapses mediated by GABA. We studied responses of $GABA_A$ receptor channels in outside-out membrane patches from cultured hippocampal neurons using rapid application techniques.[1] Patches were voltage-clamped at -60 mV using a CsCl-based pipette solution, and were exposed to short and long pulses of GABA (10-300 μM, 1-400 ms, n > 4 for each condition). The current onset (10-90% rise time^{-1}) increased with GABA concentration, and this relationship was empirically fitted to the Hill equation with a maximum of 452 ± 47 s^{-1} (mean ± SD), a K_D of 154 ± 27 μM, and a coefficient of 1.4 ± 0.2 (r = 0.99). Long pulses of high concentrations of GABA (400 ms, 100-300 μM) revealed biexponential desensitization (time constants ~20 ms and ~1 s). Upon removal of GABA, deactivation was also biexponential (27 ± 14, 186 ± 60 ms). Short pulses (1-10 ms, 300 μM, see Figure) produced currents that peaked in < 3 ms and decayed with two time constants similar to deactivation following long pulses, and to $GABA_A$ receptor-mediated synaptic currents in hippocampal cultures (~20 and ~80 ms).[2] These results imply that a synaptic timecourse of GABA as brief as a few milliseconds is sufficient to account for the shape of the synaptic current.

1) Science, 258:1498-1501; 2) J. Neurophysiol. 70:1339-1349.

Figure:

The ensemble current response to a 2 ms pulse (n = 68) of 300 μM GABA. The 10-90% rise time is 1.7 ms. Decay time constants are 16 ms (58% amplitude) and 113 ms.

Th-Pos452

THE VOLTAGE DEPENDENCE OF THE MECHANOSENSITIVE CHANNELS OF BACILLUS SUBTILIS IS ALTERED BY DNA
((I.Szabo', C.Berrier[1], A.Ghazi[1] and M.Zoratti)) CNR Unit for the Physiology of Mitochondria, Padova, Italy and [1]CNRS Unit for Biomembranes, Orsay, France. (Sponsored by M.Zoratti)

The membrane of B.subtilis contains high-conductance stretch-activated channels, whose open probability depends exponentially on the applied potential, increasing e-fold every 13 mV (Szabo' et al., Biochim. Biophys. Acta 1112, 29-38 (1993)). When bacterial DNA is added to the cytoplasmic side of an excised inside-out patch, in the positive voltage range channel behavior remains the same, but at negative potentials the open probability increases exponentially with the absolute value of the potential We tentatively interpret this change as being due to the interaction of DNA, driven to the membrane by negative potentials, with the channels.

We have compared the channel activity of wild-type cells and of cells carrying disruptions of the com locus (courtesy of Dr. Dubnau). The latter exhibited channels similar to those of the wild-type. Nonetheless, the possibility ought to be considered that these channels might play a role in bacterial transformation.

Th-Pos453

MODIFICATION OF KAINATE INDUCED INCREASES IN INTRACELLULAR FREE CALCIUM AND THEIR RELATIONSHIP TO DELAYED TOXICITY IN CEREBELLAR GRANULE CELL CULTURES. ((A.F. Strautman, H.M. Scherch, and P.F. VonVoigtlander)) CNS Diseases Research, The Upjohn Co., Kalamazoo, MI 49001.

Increases in intracellular free calcium concentration ($[Ca^{+2}]_i$) have been shown to be related to toxicity in many primary CNS cultures (Dubinsky, J.M., 1993). We have developed a method to correlate changes in $[Ca^{+2}]_i$ as measured with a fura 2 imaging system and toxicity determined at a single cell level. In particular, we are studying the prolonged elevation of $[Ca^{+2}]_i$ following pulsitile exposure to kainic acid. There appears to be a characteristic post washout pattern that indicates whether the cell will be alive when assayed 24 hours after agonist application. We are using pharmacological approaches including voltage and receptor activated Ca^{+2} channel blockers, ion substitution, and the voltage-gated sodium channel blocker U-88958, in an effort to affect changes in $[Ca^{+2}]_i$ that are induced by kainate. In the absence of extracellular Ca^{+2}, kainate induces no increases in $[Ca^{+2}]_i$ nor does it cause toxicity. Nifedipine improves survival and decreases the post washout sustained elevation of $[Ca^{+2}]_i$. Although Aga IVA, a P-type Ca^{+2} channel blocker, did not protect against kainate toxicity, U-88958 did.

Th-Pos454

MOLECULAR MODELING OF LIGAND INTERACTIONS WITH THE cGMP BINDING SITE FROM RETINAL CHANNELS ((S-P Scott, A.J. Epstein, M.C. Lopez, and J.C. Tanaka)) Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa., 19104

The nucleotide gated channel from retina is selectively activated by cGMP with a $K_{0.5}$ of ~20 μM which is 100 fold less than the $K_{0.5}$ for cAMP. Since the nucleotides differ only at the 2 and 6 positions of the purine, the amino acids which interact with these positions must encode ligand specificity. Recently, Kumar and Weber (KW) constructed a 3-dimensional model of the cGMP binding site of the bovine retinal channel (*Biochemistry*, 1992, 31, 4643). We have employed standard molecular modeling techniques to construct models of nucleotide analogs in order to visualize the molecular interactions between these ligands and the binding site. Each analog was docked into the binding site of the KW model so that steric, van der Waals and coloumbic interactions could be examined.

Sterically, all active ligands are accommodated in the binding site except for a large, active fluorescein derivative. Modeling predicts the loss of a hydrogen bond between Thr 560 and the 2 position of cAMP. A variety of substituents are tolerated in the 6 position which interact with Phe 533 on ß sheet 5. Certain substituents also interact with Asp 604 on alpha helix C. The substituents on the 8 position interact with the alpha helix C and the ß sheets 6 and 7. Models of Thr560Ser and Thr560Ala mutant channels (Altenhofen, W. et. al., *PNAS*, 1991, 88, 9868) were constructed to examine the hydrogen bond between amino acid 560 and the 2 position. The models showed a more favorable hydrogen bond interaction between cGMP and the serine mutant as compared to the wild type or the alanine mutant.

Supported by NIH EY06640.

CHANNELS IN SMOOTH MUSCLE FUNCTION

Th-Pos455

BILE ACIDS DIRECTLY ACTIVATE LARGE CONDUCTANCE Ca^{2+}-ACTIVATED K^+ (CAK) CHANNELS IN VASCULAR SMOOTH MUSCLE CELLS. ((Alejandro M. Dopico, John V. Walsh, Jr. and Joshua J. Singer)) Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01655. (Spon. by S.N. Treistman)

The effects of bile acids and synthetic analogs on CAK channel activity were studied in single smooth muscle cells freshly isolated from the rabbit proximal mesenteric artery. Cholic, deoxycholic, and lithocholic acid (30-100 μM in application pipette) reversibly activated CAK channels without changing the unitary conductance of the channel. Activation was repeatedly observed when compounds were applied to the membrane external to the patch in cell-attached patches (130 mM extracellular K^+) or when applied to the internal surface of inside-out patches when both surfaces were bathed with a 0 Ca^{2+} solution containing 5 mM EGTA. Similar activation was seen after the application of either cholic acid methyl ester or 5β-cholan-3α,7α,12α,24-tetrol, which indicates that the carboxylate group in the lateral chain is not necessary for the activation of CAK channels. Lithocholic hemisuccinate also increased channel activity, but 5β-cholanic acid failed to do so, suggesting that polar groups attached to the steroid nucleus are necessary for the activation. In addition, 3β-hydroxy-cholanic acid did not activate CAK channels whereas the α-isomer (lithocholic acid) did. Lithocholic tauroconjugate (which is unable to "flip" across the bilayer) was effective in rapidly activating CAK channels when applied to the internal surface of inside-out patches but failed to do so when applied to cell-attached patches. These results suggest that the access of bile acids to a site located on or close to the channel protein is necessary for the activation of CAK channels and may explain vasodilation caused by these steroids.

Th-Pos456

ANALYSIS OF ION CURRENTS MEDIATING MODULATION OF CONTRACTIONS OF THE ARC MUSCLE OF *APLYSIA* BY SIMULTANEOUS ON-LINE LENGTH MEASUREMENT AND CURRENT/VOLTAGE CLAMP. ((V. Brezina and K. R. Weiss)) Dept. of Physiology and Biophysics, Mt. Sinai School of Medicine, CUNY, New York, NY.

In response to behavioral demands, ACh-induced contractions of the ARC muscle of *Aplysia* are modulated by serotonin (5-HT) and a variety of peptide cotransmitters released from the muscle's own motorneurons, among them the small cardioactive peptides (SCPs), myomodulins (MMs) and FMRFamide-related peptides (FRPs). The SCPs, 5-HT and MM_B predominantly potentiate the contractions, the FRPs depress them, and MM_A both potentiates and depresses. Previously, working with single dissociated ARC muscle fibers, we found that this pattern of effects on contractions is reproduced in the pattern of the modulators' effects on two ion currents: the potentiating modulators enhance the 'L'-type Ca current, while the depressing modulators activate a large K current. We have now combined current and voltage clamp (CC, VC) with on-line video measurement of contractions of the single fibers to obtain the following direct evidence that these actions on ion currents indeed underlie the modulation of contractions: (1) Ca^{2+} entry through the 'L'-type Ca channels is essential for normal contraction. Depolarization beyond the activation threshold of the Ca current is both necessary and sufficient for contraction, equally with ACh application and CC/VC steps in the absence of ACh. All such contractions cease in Ca^{2+}-free solution or when the Ca channels are blocked with nifedipine. (2) All contractions involving activation of the Ca current, whether due to ACh or CC/VC steps, are equally potentiated by the potentiating modulators, whereas contractions that bypass its activation, e.g. induced by the Ca^{2+}-ionophore A23187 or caffeine, are not. (3) Depression of ACh or CC contractions is associated with a reduction in the depolarization attained, and thus the degree to which the Ca current is activated. This reduction is both sufficient and necessary (VC contractions are not depressed). Both the reduction in depolarization and the depression of contractions are blocked by low-micromolar 4-AP, which selectively blocks the modulator-activated K current. It thus appears likely that the enhancement of the 'L'-type Ca current is in large part responsible for the potentiation, and activation of the 4-AP-sensitive K current for the depression, of ARC-muscle contractions by the SCPs, MMs, FRPs and 5-HT.

Th-Pos457

PARADOXICAL EFFECTS OF CYCLOPIAZONIC ACID ON MEMBRANE CONDUCTANCES IN CANINE COLONIC MYOCYTES. ((A. Carl, O. Bayguinov and K.M. Sanders)) Department of Physiology, University Nevada School of Medicine, Reno, NV 89557, USA,

Cyclopiazonic acid (CPA) is a new and selective inhibitor of the sarcoplasmic Ca^{2+}-ATPase. We investigated the action of CPA on electrical slow wave activity of the circular muscle layer of canine colon and on membrane currents of dispersed colonic myocytes. CPA (10^{-5} M) increased slow wave duration from an average of 6.5±1.2 sec to 38.3±4.2 sec (n=3). In order to test whether this increase was due to an increase in Ca^{2+} conductances or decrease of K^+ conductances we measured membrane currents under whole cell voltage clamp in dialyzed and non-dialyzed (amphotericin permeabilized) myocytes. CPA (10^{-5} to 10^{-4}M) had little effect on membrane currents in cells dialyzed with 1 mM EGTA in the pipette solution. When amphotericin was used, outward currents had a large, noisy transient component (I_{TO}) that was due to activation of Ca^{2+}-activated K^+ channels. CPA (10^{-5} M) decreased this current to 68±10% of control. Under Ca^{2+}-free conditions (Ca^{2+} in bath replaced by 2 mM Mn^{2+}) outward currents were much smaller and CPA (10^{-5} M) slightly increased Ca^{2+}-activated K^+ current at potentials positive of +30 mV, indicative of an increase in basal intracellular free Ca^{2+}. Using Cs^+-filled amphotericin backfilled pipettes we recorded stable L-type Ca^{2+} currents. CPA (10^{-5} M) increased peak Ca^{2+} current 2.4±0.8fold but not the sustained inward current after >100 ms pulse duration. (NIH-DK 41315)

Th-Pos458

CHOLINERGIC SUPPRESSION OF Ca^{2+} CURRENT IN GASTRIC SMOOTH MUSCLE. ((Gregory R. Wade and Stephen M. Sims)) Dept. of Physiology, Univ. of Western Ontario, London, Ontario Canada, N6A 5C1. (Spon. by M.H. Sherebrin)

Acetylcholine (ACh) causes depolarization of visceral smooth muscle cells which is thought to elicit voltage-dependent Ca^{2+} current (I_{Ca}) contributing to elevation of $[Ca^{2+}]_i$. ACh also causes release of Ca^{2+} from intracellular stores, which directly regulates the activity of various ion channels and initiates contraction. We investigated effects of ACh on I_{Ca} in freshly isolated guinea-pig gastric smooth muscle cells using the nystatin perforated-patch technique. L-type Ca^{2+} currents were identified based on their sensitivity to dihydropyridines and voltage-dependence of activation and inactivation. ACh caused contraction and activated non-selective cation current in cells held under voltage clamp. When cells were periodically depolarized from -60 mV to 0 mV, ACh also caused a rapid reduction in I_{Ca} (mean reduction ± S.E.M., 36±3%, n=12) with recovery typically within 30 s. We tested the hypothesis that the reduction of I_{Ca} was due to Ca^{2+}-dependent inactivation of I_{Ca} mediated by release of Ca^{2+} from intracellular stores. Caffeine inhibited I_{Ca} to a level similar to that caused by ACh (36±4% reduction, n=7). When Ba^{2+} was used as the charge carrier in a Ca^{2+}-free solution, ACh also inhibited Ca^{2+}-channel current (54±6% reduction, n=7). Thus, Ca^{2+} influx is not required for the suppression of I_{Ca} by ACh. These findings are consistent with cholinergic release of Ca^{2+} from intracellular stores mediating Ca^{2+}-dependent inactivation of I_{Ca}. Such inhibition of I_{Ca} would reduce Ca^{2+} entry during cholinergic depolarization of smooth muscle cells.

Supported by The Ontario Thoracic Society and Medical Research Council (Canada).

Th-Pos459

STRETCH-INDUCED Ca^{2+} ENTRY IN A7r5 AORTIC SMOOTH MUSCLE CELLS. ((V. Ruiz-Velasco, B.M. Mayer and L.J. Hymel)) Dept. of Physiology, Tulane University School of Medicine, New Orleans, LA 70112.

An increase in arterial pressure initiates a sequence of events that leads to contraction of vascular smooth muscle cells (SMC) caused by an increase in intracellular $[Ca^{2+}]$. In order to study how stretching aortic SMC elicits a contractile response, we used the A7r5 SMC line derived from rat thoracic aorta to measure ^{45}Ca uptake under stretched and nonstretched conditions. The cells were seeded at 5000 per cm^2 in 25 mm wells containing a collagen-coated silastic substratum, and were grown for 10 days to a confluent monolayer prior to assay. The plates were then mounted in the Flexercell Strain Unit (Flexcell Int. Corp., McKeesport, PA), and the substratum was stretched and relaxed using a defined negative pressure that achieved an average of 10% surface elongation in a 6 second cycle (3s on, 3s off). $^{45}Ca^{2+}$ uptake was measured at 1, 2, 3, 5, 10, and 30 min under resting (5 mM K^+) and depolarizing (50 mM K^+) conditions in the presence or absence of 1 μM (+)-isradipine. When the cells were stretched at 5 mM K^+, Ca^{2+} uptake increased significantly compared to the nonstretched cells (initial rate = 1.17 nmoles/(min x 10^6 cells) stretched vs. 0.24 nonstretched). Depolarizing conditions (50 mM K^+) without stretch stimulated Ca^{2+} uptake to similar levels (0.89 nmoles/(min x 10^6 cells)) as the application of stretch at 5 mM K^+, but did not further stimulate Ca^{2+} uptake with stretch (0.98). Both the K^+- and stretch-induced Ca^{2+} entry were effectively blocked (88-94%) by isradipine. These results suggest that stretching A7r5 cells at 5 mM K^+ stimulates Ca^{2+} entry by causing membrane depolarization and subsequent activation of L-type Ca^{2+} channels, or by direct activation of L-type Ca^{2+} channels. Supported by a Grant-In-Aid from the American Heart Association, La. Affiliate Inc.

Th-Pos460

OSMOTICALLY SENSITIVE WHOLE CELL Ba^{2+} CURRENT AND Ca^{2+}-DEPENDENT FORCE RECORDED FROM RAT BASILAR ARTERY. ((P.D. Langton & N.B. Standen)) Ion Channel Group, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, U.K.

Recently we have found that changes in bath osmolarity result in profound but reversible changes in Ba^{2+} (10mM) current magnitude. Superfusate osmolarity was altered from control (292mosmol l^{-1}) by omission of NaCl (30mM; hyposmotic, 238mosmol l^{-1}) or addition of sucrose (60mM; hyperosmotic, 354mosmol l^{-1}). For a series of nine cells the peak inward current was -78pA (±17, 9) which increased to -125pA (±18, 5) and declined to -40pA (±6, 4) during hypo- and hyper-osmotic superfusion, respectively (mean (±S.E., n)). Activation curves constructed from these data were fit with Boltzmann functions to give values of half activation of -5.7±1.4, -8.9±2.1 and -0.2±0.3mV for control, hypo- and hyper-osmotic solutions, respectively. No effects on other conductances, nor capacitive surface area were observed and the sensitivity of the Ba^{2+} current to the dihydropyridine (DHP) antagonist (-)202-791 was unchanged.
Because force in cerebral arteries is highly sensitive to DHP antagonists (K_d 2.4nM, n=8), we examined the effect of similar alterations in osmotic strength on high $[K^+]_o$ (40mM) induced isometric force. Relative steady-state force developed in response to high K^+ (40mM) was reversibly potentiated to 1.43±0.043 and depressed to 0.46±0.021 of control values by hypo- and hyper-osmotic solutions, respectively (n=14 tissues). These data show changes DHP-sensitive Ca current, observed in isolated myocytes, in response to changes in osmotic strength. In addition, force measurements are consistent with a similar modulation of Ca^{2+} channel function in intact tissue. These findings may indicate a role for mechanical stress in the regulation of voltage-dependent ion channel function.
Supported by the British Heart Foundation.

Th-Pos461

BLOCK OF VOLTAGE-GATED CA^{2+} CHANNELS IN RAT VASCULAR SMOOTH MUSCLE BY AN ALKYLAMMONIUM DERIVATIVE. ((Boyle, J.P. and Langton, P.D.)) Department of Cell Physiology & Pharmacology, University of Leicester, Leicester, LE1 9HN, U.K.

Alkylammonium ions have long been known to block certain K^+ and Na^+ channels and have been used, in the bis-form, to map the dimensions of a K^+ channel (Villeroel et al,(1988), Pflügers Arch, 413, 118). We report here the effects of bis-TPeA (1,4-bis(tripentylammonium) butane dichloride) on the tone of, and voltage-gated Ca^{2+} channels in, rat vascular smooth muscle. In vitro, bis-TPeA caused a concentration-dependent suppression of noradrenaline- or 80mM K^+-supported tone in rat mesenteric artery (IC_{50} 4.2± 0.6μM) and rightward shift of the concentration-effect curve for Ca^{2+} induced spasm in depolarized tissue using a calcium re-entry protocol. These results suggest that bis-TPeA is blocking Ca^{2+} influx through voltage-gated channels. This was investigated using whole-cell recording of Ba^{2+} currents (I_{Ba}) in isolated rat basilar artery myocytes. Bis-TPeA caused a concentration-dependent block of both peak and sustained inward currents evoked by voltage-clamp steps from a holding potential of -88mV to -40 to +10mV. These blocking effects were rapid suggesting an external site of action but were not well fit by a simple fractional occupancy model. Bis-TPeA blocked a non-inactivating component of I_{Ba}, and this block showed no appreciable voltage dependence. The concentrations of bis-TPeA causing 50% block of peak and sustained currents were 300 and 100μM respectively. In conclusion we have shown that bis-TPeA blocks the voltage-gated Ca^{2+} channel/s responsible for both agonist- and depolarization-induced Ca^{2+} influx in rat vascular smooth muscle.
Supported by The British Lung Foundation and The British Heart Foundation.

Th-Pos462

REGULATION BY OVARIAN HORMONES OF THE LARGE-CONDUCTANCE Ca^{2+}-ACTIVATED K^+ CHANNELS OF FRESHLY DISSOCIATED RABBIT UTERINE MYOCYTES. **((L. Yang, S. Y. Wang and C. Y. Kao)) Department of Pharmacology, SUNY Downstate Medical Center, Brooklyn, NY 11203.**

Uterine myocytes were freshly dissociated from 2-month old immature virgin rabbits, or after treatment with estrogen, or with additional progesterone, or with concomitant tamoxifen. In detached inside-out patches in asymmetric K^+ solutions (in/out: 135/4.5), 200 pS, voltage-dependent, Ca^{2+}-sensitive channels (maxi-K^+) are seen among other smaller and rarer openings. The voltage-dependence of open-probabilities (np_o) of the maxi-K^+ channels follows Boltzmann distributions. In untreated myocytes at pCa 8 and 7, the np_o are 74 and 54 mV, with slopes (k) of 9.2 and 8.8 mV. In estrogen-treated myocytes at the same pCa's, np_o's are 53 and 48 mV; k's, 9.5 and 9.4 mV, showing a significant (t-test, $p<0.05$) rise in Ca^{2+} sensitivity at low $[Ca^{2+}]_i$, which is masked by higher $[Ca^{2+}]_i$. In progesterone-treated myocytes, np_o's are 78 and 43 mV; k's 9.5 and 9.4 mV, showing a significant ($p<0.05$) anti-estrogen effect at low $[Ca^{2+}]_i$. In tamoxifen-treated myocytes, although np_o at pCa 8 is 53 mV, k is 7.5 mV with most shifts at high positive voltages, suggesting that tamoxifen interferred with the genomic influences of estrogen on synthesis of new channels, but not with its regulatory influences on channel expression. **(Supported by NIH grant HD00378).**

Th-Pos463

EFFECTS OF TACHYKININE ON INWARD CURRENTS OF CANINE COLONIC MYOCYTES. ((Hye Kyung Lee and Kenton M. Sanders)) Dept. of Physiology, Univ. of Nevada, Reno, Nevada 89557.

Substance P (SP) and neurokinin A (NKA) prolong electrical slow waves and depolarize canine colonic muscle. The ionic mechanism underlying these effects was studied in isolated myocytes using the whole cell patch clamp technique at 33 ± 1°C. K^+ currents were minimized by internal dialysis with Cs^+, and the Cl^- gradient was adjusted to E_{Cl} = -70 mV. SP and NKA activated a noisy inward current (I_{NKA}, I_{SP}) at -70 mV with amplitudes of 24 ± 3 pA (n=13, 10 μM SP), 28 ± 3 pA (n=9, 0.1 μM NKA). I_{NKA} and I_{SP} were nonselective to cations such as Na^+, Li^+. I-V relationships were linear and reversed around 0 mV. Under current clamp, NKA (0.1 μM) depolarized membrane potential. Reduction of extracellular Na^+ from 130 to 35 mM by replacement with NMDG reversed this depolarization. I_{SP} and I_{NKA} were inhibited by Cd^{2+} (0.5 mM, n=2) and quinine (1 mM, n=3). In Ca^{2+}-free bath solution, NKA (0.1 μM) failed to induce I_{NKA} but subsequent addition of Ca^{2+} to the bath activated the current, suggesting Ca^{2+} is required to initiate I_{NKA} or I_{SP}. However membrane depolarization and activation of Ca^{2+} current did not further facilitate I_{NKA} or I_{SP}. In addition, NKA (0.1 μM) also inhibited L-type Ca^{2+} current by mechanisms which appear to be both Ca^{2+}-dependent and -independent. (Supported by DK 41315).

Th-Pos464

HYDRALAZINE RELAXES RABBIT AORTA BY BLOCKING INTRACELLULAR CALCIUM RELEASE ((M. Allam and A.M. Gurney)) Department of Pharmacology, UMDS, St. Thomas's Hospital, London, U.K. SE1 7EH

The vasodilator hydralazine has been used clinically for over 40 years, but its actions are poorly understood. Strips of rabbit aorta precontracted with 1μM phenylephrine relaxed by 78±4% (n=8) in response to 100μM hydralazine. Relaxation took up to 1 hr to develop fully and was often preceded by a brief contraction. Hydralazine did not block voltage-gated calcium channels, because relaxations were unaffected by the calcium antagonist diltiazem (8μM) and hydralazine did not relax tissue precontracted by depolarisation with 50mM external K^+. The relaxation induced by hydralazine (100μM) was also unaffected by cyclopiazonic acid (30μM), thapsigargin (1μM) and ryanodine (10μM) when applied on their own. However, when cyclopiazonic acid and diltiazem were applied together, the combined effect was to reduce the hydralazine-induced relaxation to only 18±9% (n=3). Cyclopiazonic acid blocks the Ca^{2+} -ATPase in the sarcoplasmic reticulum (SR) that sequesters Ca^{2+} from the cytoplasm. Ca^{2+} has also been suggested to enter the SR through a separate pathway that is sensitive to Ca antagonists. Thus, in combination, these drugs would effectively deplete the SR of Ca^{2+}. Under these conditions, Ca^{2+} contributing to tension would originate primarily from extracellular sources and not the SR. Since, under these conditions, hydralazine became relatively ineffective, we conclude that it relaxed the muscle by blocking Ca^{2+} release from intracellular stores. A mechanism involving the Ca^{2+} stores was further suggested by the finding that hydralazine (100μM) caused a 23±6% (n=4) inhibition of contractions induced by caffeine (10mM) and 85±6% (n=3) inhibition of contractions induced by phenylephrine (1μM) in Ca^{2+}-free solution.

Th-Pos465

SITE-DIRECTED BIOTINYLATION USED TO IDENTIFY A VOLTAGE-DEPENDENT CONFORMATIONAL CHANGE IN THE COLICIN IA CHANNEL. ((Stephen Slatin, Karen Jakes, Xiao-Qing Qiu, Alan Finkelstein)) Dept. of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, N.Y. 10461

Channel-forming colicins are *E. coli* proteins that form voltage-dependent channels in membranes and are lethal to sensitive strains of *E. coli*. Experiments with colicin E1 have led to a model of voltage dependence based on the insertion of alpha helical segments into the membrane in response to *cis* positive voltage. The partly homologous protein colicin Ia is thought to gate by a similar mechanism, and offers certain advantages as a model channel. Here, we determine the location of cysteine residues introduced into colicin Ia by site-directed mutagenesis, to examine the structure of the open and closed channel. Residues 537, 540, 541, 544 and 547 were independently mutated to cysteine, and the resulting unique cysteine residue was biotinylated with an NEM-biotin reagent. For each of these 5 biotinylated colicins, the biotin-binding protein streptavidin was found to block its killing of sensitive cells by one to two orders of magnitude. All five proteins formed channels in planar bilayers that were similar to wt channels, and in each case, streptavidin added to the *cis* chamber prevented closed channels from reopening. Thus, the biotin at each of these loci was accessible to the *cis* side of the membrane in the closed state. Streptavidin added to the *trans* chamber had no effect on two of the constructs, but for the other three (537-, 540- and 541-biotin), it interfered with the closing of channels that were exposed to it in the open state. Taken together, these results show that this small region of the protein (residues 537-541) moves from a position at or near the *cis* side of the membrane to at or near the *trans* side of the membrane in conjunction with channel gating. These results are consistent with the insertion model of gating. (supported by NIH 29210-15)

Th-Pos466

CHARACTERIZATION OF ELECTROSTATIC INTERACTIONS IN INITIAL BINDING OF COLICIN E1 CHANNEL DOMAIN TO MEMBRANE VESICLES ((Y.-L. ZHANG, J. B. HEYMANN, AND W. A. CRAMER)) Dept. of Biological Sciences, Purdue University, West Lafayette, IN 47907

Association of the colicin E1 channel domain with uniformly sized DMPG/DOPE large unilamellar vesicles was studied by nonradiative fluorescence energy transfer, from the three intrinsic tryptophans in a 187 residue C-terminal tryptic channel peptide fragment, to trinitrophenyl conjugated to PE in vesicles doped with 10% TNP-PE. A major role of electrostatic interactions in the initial binding of the fragment to the membranes was inferred from: (i) dependence of the binding capacity (lipid molecules/channel domain) of the vesicles on (a) ionic strength (0.125-0.3 M) of the reaction medium, (b) net positive charge of the fragment (at pH 3,4), and (c) negative surface charge density (DMPG content) of the vesicles. The K_d was 2.0×10^{-9} M and 4.5×10^{-9} M, and the binding capacity 250 and 360 lipids/channel fragment, at ionic strengths of 0.125 and 0.2 M, respectively, pH 4, and 30% DMPG. Mutagenesis of the residues Lys362 and Lys403, suggested by analogy with colicin A to be important in docking to the membrane, to Ile362/Ile403 yielded an altered fragment whose binding was characterized by a similar affinity but a higher capacity, implying a different binding conformation. The high affinity of the positively charged channel domain for negatively charged membranes might be partly attributed to the involvement of hydrophobic interactions subsequent to the initial contact and binding, because the net binding was not reversible upon shifting the pH from 4 to 8 in the presence of high ionic strength. (We thank T. Walter for making the double K->I mutant; supported by NIH GM-18457).

Th-Pos467

INACTIVATION OF COLICIN CHANNELS WITH SMALL VOLTAGES IS AFFECTED BY AN N-TERMINAL DOMAIN. ((D.C. Ok., F.S. Cohen, Y.-L. Zhang, and W.A. Cramer)) Rush Medical College, Chicago, IL 60612 and Purdue University, W. Lafayette, IN 47907. (Spon. by J. Hirsh).

It is recognized that colicin channels inactivate with large activating voltages, but not realized that they also inactivate with small voltages into an energetically more shallow state from which they easily recover. After activating colicin E1 in voltage-clamped planar membranes with small voltages, switching to deactivation voltages results in more instantaneous current than expected from a ratio of the voltages. The single channel I-V relations are linear, consistent with the extra current due to fast recovery from inactivation. With C-terminal peptide this extra current is not observed. Also, tail currents are faster and more voltage dependent for peptide than for intact E1. We conclude that the N-terminus of the protein promotes inactivation, recovery leads to slower tail currents, and recovery reduces the apparent voltage-dependence of deactivation. With increased time, tail currents become slower with colicin E1 but do not change with peptide. This suggests that an N-terminal domain increasingly interacts with the channel over time, leading to energetically deeper inactivated states. N-terminus-induced inactivation is reminiscent of the inactivation mechanism proposed by others for Na and K channels. Supported by NIH GM27367 and GM18457.

Th-Pos468

GATING AND IONIC CURRENTS OF VOLTAGE-GATED IONIC CHANNELS EXPOSED TO GENERAL ANESTHETICS ((A. M. Correa)) Dept. of Physiol., UCLA, Los Angeles, CA, 90024.

The effect of general anesthetics on the gating characteristics of voltage dependent channels was studied using the cut-open oocyte technique. Ionic and gating currents were recorded from potassium channels of the *Shaker* family expressed in *Xenopus* oocytes. The K^+ channel clones H4IR, which lacks fast inactivation, and the non conducting W434F mutant of the H4IR were used. External and internal solutions contained 120 Na^+ or 60 Na^+-60 K^+ and 120 K^+, respectively. The results presented here were obtained with chloroform and isoflurane, a volatile anesthetic. Anesthetics were perfused through in the external medium. Both compounds modified ionic and gating currents. Chloroform, at approx. 60 mM, depressed K^+ currents by as much as 85%. Currents were slightly slower than controls. Chloroform did not affect the response to hyperpolarizing prepulses (Cole-Moore shift). In the W434F, 'on' gating currents peaked earlier and decayed as the controls. No obvious differences were found in the Q-V curves. In contrast, 'off' gating currents were dramatically accelerated (3-5 fold); the effect was more evident at positive potentials and was reversible. In isoflurane (3%) the ionic currents were slower than the controls, in particular, the tail currents. Both 'on' and 'off' gating currents were modified. Currents were smaller and also, at potentials more positive than -20 mV, there was an additional slowing down in the return of the charge as if isoflurane caused some charge immobilization. At positive potentials, an apparent shift in the voltage dependence of activation was seen. The results with these and other anesthetics tested seem to indicate specificity in general anesthetic effects. Supported by USPHS GM30376.

Th-Pos469

SLOWING OF DEACTIVATION KINETICS IN *SHAKER B* AS SEEN IN MACROPATCH RECORDINGS OF GATING AND IONIC CURRENTS. ((D. Sigg*, F. Bezanilla*, E. Stefani§)) *Department of Physiology, UCLA, Los Angeles, CA 90024 and §Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston TX.

We have studied the effects of macropatch excision on *Shaker B* deactivation kinetics. In the cell-attached mode, it is possible to obtain records of gating currents from *Xenopus* oocytes injected with the non-conducting *Shaker* H4-IR W434F K^+ channel that are very similar to those from whole-cell records obtained with the cut-open oocyte technique (Bezanilla et. al., *Science* 254: 679, 1992). However, within minutes after excision, the time constant of the decay phase of deactivation (OFF) gating currents gradually increases by about 3-fold while the kinetics of the activation (ON) current remains similar or becomes slightly faster. By varying the value and duration of the depolarizing pulse, we can associate the slowing of OFF kinetics with components of the gating current associated with transitions near the open state of the channel. Accordingly, records of ionic deactivation currents from the conducting *Shaker* H4-IR channel exhibit slowing over a similar time course as those from OFF gating currents. Re-introduction of the excised patch into the intracellular milieu of the oocyte does not recover the kinetics seen before excision. The effect is not restricted to excised patches, as the selective slowing of deactivation kinetics can be observed on a slower time scale even in cell-attached patches, and the initial kinetics of the OFF gating current varies from batch to batch of oocytes taken from different frogs. Supported by USPHS GM30376 and AR38970.

Th-Pos470

AN ELECTROCHEMICAL GATING MODEL FOR EFFECTS OF Rb^+ AND K^+ ON INWARD RECTIFIER K-CHANNELS. ((P.Pennefather[1] and T.DeCoursey[2]))[1]Fac. Pharmacy, Univ. of Toronto, Toronto, ON, Canada, M5S 2S2 ,[2]Dept. Physiology, Rush Medical Center, Chicago, IL., 60612.

The effects of replacing K^+ with Rb^+ on the kinetics and steady-state activation of an inwardly rectifying K-channel (IR) found in bovine artery endothelial cells place constraints on models of gating of that channel. Our model proposes that both Rb^+ and K^+ act as allosteric modulators of an intrinsically voltage dependent isomerization between open and closed states. Occupancy of binding sites on the outside of the channel promotes channel opening and stabilizes the open state. In addition, Rb^+ can plug the pore of the IR channel. Occupancy of the pore by Rb^+ can modify the rates of isomerization and the affinity of the allosteric sites for activator ions. The model incorporates the proposed triple barreled nature of the IR channel by proposing that plugging of the channel is a cooperative process involving a single site in each of the three bores. Interaction between bores during plugging and permeation is consistent with correlated flux models of IR conductance. Parallel bores multiply the number allosteric sites associated with the macromolecular channel and allow for steep voltage dependence without compromising the parallel shift of the half-activation potential with reversal potential. The model reproduces experimental observations provided channel plugging by Rb^+ is associated with: decreased isomerization rates, increased affinity of allosteric sites on closed channels and, decreased affinity of sites on open channels. Rb^+ also slows closing at potentials were little plugging is expected suggesting Rb^+ is 1.5x more potent then K^+ as an activator. Since Rb^+ has a lower permeability then K^+, cation binding sites that regulate gating probably are different from those governing permeation.

Th-Pos471

IONIC CURRENTS IN RABBIT RETINAL PIGMENT EPITHELIAL CELLS. ((Q-P. Tao, J.F. Poyer and M.E.M. Kelly)) Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4H7.

Ionic currents were studied in isolated rabbit retinal pigment epithelial (RPE) cells using whole-cell recording. RPE cells exhibited both inward and outward voltage-dependent currents. When K^+ was the principal cation in the recording electrode, outward K^+ current was apparent in 91% of cells. Pharmacological and kinetic analysis identified 2 distinct K^+ currents, a time- and voltage-dependent outward current, which resembled the delayed rectifier K^+ current described in other cell types, and an outward current which decayed rapidly at positive potentials. In 5 mM K^+ bathing solution, K^+ tail currents reversed at -73±6 mV (n=4), with the reversal potential shifting 50 mV/10-fold change in $[K^+]_o$. Outward K^+ current was reduced 57% (n=6) and 38% (n=4) by external tetraethylammonium ions (1 mM) and Ba^{2+} (1 mM) and abolished by 1 mM quinine. Inwardly rectifying K^+ current was also present in 41% of cells and was blocked by extracellular Ba^{2+} and Cs^+ and exhibited Na^+ dependent inactivation at negative potentials. In some cells, when I_K was blocked with Cs^+ in the pipette and extracellular Na^+/K^+ replaced with $NMDG^+$, a current was observed which reversed around 0 mV under symmetrical ionic conditions. In other cells, current was only observed when RPE cells were exposed to either caffeine or hypoosmotic Ringers. In 3 cells tested, 30% of this induced current was inhibited by niflumic acid. The niflumic-sensitive current had a reversal potential close to E_{Cl}. We conclude that rabbit RPE cells express at least 3 voltage-dependent K^+ currents. Rabbit RPE cells may also exhibit an anion-selective current which is sensitive to $[Ca^{2+}]_i$. Both the K^+ and anion conductances reported here may provide conductive pathways that support electrogenic ion transport and volume regulation in the RPE.

Supported by NSERC OGP0121657 and Retinitis Pigmentosa, Canada (Q-PT).

Th-Pos472

IDEALIZATION OF ION CHANNEL RECORDS BY STUDENT T-TEST FILTERING WITH SUBSEQUENT OPTIMIZATION OF LEVEL DISTRIBUTION LIKELIHOOD. ((V. Pastushenko and H. Schindler)) Inst. Biophysics, J. Kepler Univ., A-4040 Linz, Austria.

A parameter-free statistical method is presented for the recovery of the stepwise current changes in ion channel records. The method starts with the Student T-test filtering of the record as primary detector of transitions. An optimum idealized trace is then obtained by reducing the number of transitions and searching for sharpest likelihood density of level distribution. The sharpness is defined as the norm of the likelihood density. The method does not require low-pass filtering which induces loss of information at corner frequencies below a specified limit. Distinct (macroscopic) states representing ensembles of level events in records of single or several channels are detected. The method has no separate limitations for both length of levels and distance between them. Even one-point levels can be detected if they differ enough from adjacent levels, and arbitrarily close levels can be resolved, provided they are long enough. A quantitative measure for the record quality is introduced in terms of the signal/noise ratio and the average level length. The method is tested with computer simulated data and illustrated by L-type Ca-channel data. Supported by Austrian Research Funds S-6606.

Th-Pos473

DIFFERENTIAL EFFECTS OF HIGH INTENSITY ELECTRICAL FIELD ON VOLTAGE-GATED Na AND K CHANNELS. ((W. Chen and R.C. Lee)) Department of Plastic and Reconstructive Surgery, The University of Chicago, Chicago, Illinois 60637

An improved double vaseline-gap voltage clamp was used with cut twitch fibers of frog skeletal muscle to study the electro-conformational damages of the voltage-gated Na and K channels, in terms of damage threshold membrane potential, channel conductance reduction and the voltage sensitivity change. Hyperpolarized shocked membrane potentials from 200 mV to 1000 mV were delivered by the voltage clamp and the pre and post-shock I-V curves were obtained. The membrane leakage currents which includes normal and shock-induced currents, and the leakage current underneath the vaseline gap were corrected. The relative maximum channel conductances of Na and K channels were plotted as semilogarithmic scale against the test membrane potential before and after the electrical shock. The steepness of the slopes during the small depolarization for the K channel showed little shock-effect, while that of the Na channel was reduced from 4mV to 5.5 mV/e-fold increase of g_{Na} after 4 shock pulses of -800 mV, 4 ms. The data was fitted by Boltzmann equation with the classic two-state mode, the equivalent moveable charged particles, z_g, for the Na channel was reduced by 20-30% therefore, some gating charged particle lost their moveability by electric shock.

Combining with the reduction of the ionic selectivity of the K channel which results in depolarization of the membrane resting potential and the decrease of the voltage sensitivity of Na channels, the electro-conformational damage of membrane proteins plays an important role in dysfunction of muscle and nerve in electrical injury.

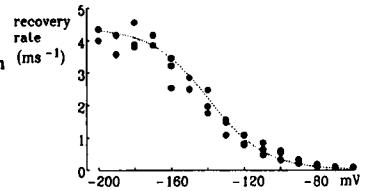
Th-Pos474

SODIUM CHANNELS MUST DEACTIVATE TO RECOVER FROM INACTIVATION

((Chung-Chin Kuo and Bruce P. Bean)) Harvard Medical School, Boston, MA 02115

We studied the kinetics of recovery from inactivation of voltage-dependent sodium channels in rat hippocampal CA1 neurons. Recovery proceeded exponentially after an initial delay and was accompanied by a tiny ionic current. Both the delay and the time constant of recovery became shorter with increasing hyperpolarization over the range from -60 to -170 mV. Negative to -170 mV, the rate of recovery saturated ($\tau \sim 0.25$ msec). Recovery from block by the neutral anticonvulsant drug diphenylhydantoin was far slower than normal recovery but the pattern of voltage-dependence was very similar.

Our results suggest that analogous to the coupling between Na channel activation and the development of inactivation, recovery from inactivation is coupled to channel deactivation and thereby derives its steep voltage dependence.



Th-Pos475

IONIC CURRENTS AND EXCITABILITY IN SEPTAL NEURONS FROM THE TRISOMY 16 MOUSE FETUS. ((L.D. Acevedo, Z. Galdzicki, and S.I. Rapoport)) Laboratory of Neuroscience, National Institute on Aging, NIH, Bethesda, MD 20892.

Human trisomy 21 gives rise to Down's syndrome (DS), and all subjects with DS will develop Alzheimer disease (AD). The trisomy 16 mouse is an animal model for human trisomy 21 because genes on mouse chromosome 16 correspond to those on human chromosome 21. We examined the electrophysiological properties of septal and hippocampal neurons from the trisomy 16 mouse fetus and from normal diploid controls, and have reported that cultured hippocampal neurons from this mouse display anomalous electrical characteristics [Galdzicki et al., 1993, Brain Res 604:69-78].

Dissociated neurons were obtained from septal brain regions of trisomic or diploid fetuses at 16 days gestation, and cultured on poly-L-lysine-coated plastic dishes. The neurons were cultured in MEM, supplemented with 5% horse serum and 40 ng/ml 7S-NGF. These cultures survived 3 weeks or longer and contained neurons of varying size (5 to 30 μm in diameter) and morphology.

Whole-cell voltage clamp records were made from septal neurons after 9 to 16 days in culture. We compared passive membrane properties and current/voltage relations of trisomic to those of normal diploid septal neurons. We found no statistically significant difference ($p<0.05$) between the two groups in passive properties, including resting potential, input resistance, capacitance, and membrane time constant. With respect to active membrane properties, maximum inward current, maximum inward conductance, maximum outward conductance, and reversal potentials for these currents we found no difference between groups. However, the ratio of inward to outward conductance was 20% higher in trisomic neurons ($p=0.01$). This difference was due to a small population (~10% of total) of trisomic neurons having a higher than average inward conductance and an accompanying lower outward conductance. Both conditions gave rise to an abnormally high conductance ratio. This population of trisomic neurons should therefore exhibit a higher excitability than normal diploid neurons.

Th-Pos476

MODELING THE DYNAMIC FEATURES OF THE SLOW INWARD CALCIUM CURRENT: FROM HODGKIN-HUXLEY TO MARKOV CHAIN MODELS. ((D.R. Lemieux, S. Nattel and G. Lee)) Institut de génie biomédical, Université de Montréal, Montréal, H3C 3J7 et Institut de Cardiologie de Montréal, Montréal, H1T 1C8

Using the whole-cell configuration of the patch-clamp technique, single- and double-pulse voltage-clamp protocols were applied to isolated human atrial cells in order to characterize the slow inward calcium current ($I_{Ca,L}$). We first modeled $I_{Ca,L}$ using the Hodgkin-Huxley (*HH*) formalism. Sequential optimization of *HH* parameters, through a linearization of the non-linear *HH* equation, was based on measurements of peak $I_{Ca,L}$ (I_p) values, their time to peak (t_p) and current time course. The resulting model was composed of a single activation (d) of degree *3*, and two inactivation (f_1 and f_2) processes of degree 1, where $f_{1\infty} = f_{2\infty}$. This model failed to reproduce experimental $I_{Ca,L}$ traces; the theoretical window current was more than twice as large as the one obtained experimentally. Residual steady-state currents were generated when the experimental protocols used to record $I_{Ca,L}$ were applied to the model. Preliminary results showed a significant reduction of these residual currents, and of the size of the window current, when $I_{Ca,L}$ was modeled with a 12-state Markov Chain model, the activation-deactivation chain containing *3* closed states and *1* open state and the inactivation chain containing *2* inactivated states and *1* open (or close) state. Applying HH restrictions to this Markov model would correspond to a *3:2* ratio for *d:f*.

Th-Pos477

CHARACTERISATION OF *RHODOBACTER CAPULATUS* PORIN CHANNELS IN PLANAR BILAYERS.

((N.D. Bishop, E.J.A. Lea and G. Rennie*)) School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, UK. and *Unilever Research, Port Sunlight Laboratory, Wirral, L63 JW, UK.

The outer membrane of Gram-negative bacteria contains aqueous channels, porins, which aid the diffusion of small hydrophilic substances across it. *Escherichia coli*, as enteric bacteria, are able to survive a hostile environment of proteases, surfactants and drastic changes of osmotic pressure. *Rhodobacter capsulatus* is not an enteric bacterium and as such has not evolved to resist the same challenges. Porins, which have molecular weight of approximately 35 kDa, form trimeric channels with a solute exclusion limit of about 600Da. Most of them open and close in a controlled manner as a function of p.d. This latter function is little understood at present. The functional properties of single trimers of the major porin of *Rhodobacter capsulatus* strain 37b4 have been investigated in planar artificial bilayers. The trimers were inserted from one side only of a bilayer. On application of a suitable p.d. the observed trimer closes in three approximately equal steps each 468 ± 7pS (n=31) in 1M KCl, 665 ± 4pS (n=70) in 1M NaCl, 492 ± 10pS (n=81) in 1M potassium-d-gluconate. The behaviour is completely symmetrical as regards closure in response to p.d.'s of opposite polarity and is strongly cation selective. Similarities and differences between characteristics of other porins is discussed in relation to the recently published atomic resolution structures for the *E. coli* porins as well as for *Rhodobacter capsulatus* porin.

Th-Pos478

CATION CHANNEL IN THE NUCLEAR ENVELOPE OF RED BEET CELL.

((Cz. Grygorczyk and R.J. Poole)) Dept. of Biology, McGill University, Montreal. (Spon. by M. Glavinovic)

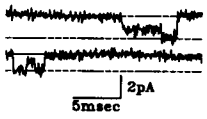
We have applied the patch-clamp technique to study ion conductances in various configurations of the red beet (*Beta vulgaris L.*) nuclear envelope. With excised inside-out and outside-out patch configurations we have observed a cation channel that was permeable for small cations (K^+, Na^+, Cs^+ and Li^+) and was activated by micromolar concentrations of Ca^{2+} on the intranuclear side of the envelope. The most prominent conductance level was 110 ±22 pS (with 150 mM KCl in the bath and pipette). The channel activity was voltage dependent and increased steeply for voltages positive on the intranuclear side of the envelope. Zn^{2+} (1 mM) when applied from cytoplasmic side of the envelope blocked the channel activity completely. In nucleus-attached configuration, following addition of the Ca^{2+} ionophore A23187 (4 μM) and 1 mM $CaCl_2$, multilevel channel openings were observed with the largest of up to 900 pS. The properties of the whole-nucleus current paralleled those observed with excised patches. These data are consistent with the channel transversing both membranes of the nuclear envelope and suggest that it may represent a regulated ion conductance pathway associated with the nuclear pore complex.

Th-Pos479

EVIDENCE FOR TWO OPEN-STATES IN CARDIAC SODIUM CHANNELS.

((S. Hirai, S. Krueger and M.F. Sheets)) Department of Medicine, Northwestern University Medical School, Chicago, IL 60611. (Spon. by M.F. Sheets)

Although multiple conductance levels in unmodified cardiac Na channels have been previously reported, it is unclear if they result from different open-states of the same channel population or from different channel populations. To investigate this question, single channel recordings in cell-attached patches that contained only a single Na channel (i.e. a probability of < 10^{-4} that more than one channel was present) were analyzed. In guinea-pig ventricular cells at 12°C (n=5), the membrane potential was stepped to test potentials (V_t) between -70 mV to -20 mV in 10 mV increments from a holding potential of -130 mV. Amplitude histograms showed at least two different conductance levels (18.6 pS and 12.1 pS) in all patches and at all V_t. The frequency of low conductance openings calculated from the ratio of area under the amplitude histogram of the low conductance openings compared to the area under both the low and normal openings was 0.20 ± 0.09 (S.D.), and did not appear to vary with V_t. There were occasional examples where one conductance level appeared to switch directly to the other level without an intervening closed state transition (Fig.). Similar results were demonstrated in guinea-pig ventricular cells at 21°C (n=2) and in cat ventricular cells at 12°C (n=2). These results suggest that unmodified cardiac Na channels have at least two different open-states and that transitions directly from one open-state to another open-state occur.



ELECTRONIC RECORDING - WHOLE CELL

Th-Pos480

INFLUENZA A M2 ION CHANNEL EXPRESSED IN *XENOPUS* OOCYTES.

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We have characterized the channel properties of the Udorn/72 Influenza A M2 channel, a member of the small (single membrane spanning) ion channel family. This channel was reported to underlie an acid-activated current when expressed in *Xenopus* oocytes (Pinto et.al. Cell 69:517-528, 1992). At lower pH's (5.5-6.5), the ohmic current has a nearly linear I-V relationship when recorded at potentials between -130 and -60 mV, and the channel is inhibited in a dose dependent manner by the anti-influenza A clinical agent, Amantadine. The current is dependent on sodium concentration, however, a direct determination of reversal potential is compromised in the whole cell configuration due to endogenous currents. Current amplitude increases as pH is lowered with an ~10 fold increase in amplitude @ pH 5.5 versus pH 8.5 when current waveforms are recorded at -120 mV. The absolute increase of current amplitude is related to the buffering capacity of the recording media likely due a control of the microenviroment pH. Relaxation of the current waveform appears to be related to level of channel expression, perfusion rate and $[Na^+]_o$.

Th-Pos481

COMPARISON OF AEQUORIN LUMINESCENCE AND ELECTROPHYSIOLOGY AS INTRACELLULAR Ca^{2+} MEASURES IN *XENOPUS* OOCYTES.

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Detection of receptor expression in *Xenopus* oocyte usually relays on functional coupling to 2nd messengers such as Ca^{2+} or cAMP. For the receptors that are coupled to elevation of intracellular Ca^{2+}, aequorin luminescence assay (Giladi E. and Spindel E., Biotech. 6:744-747, 1991) is an attractive alternative to electrophysiology because of its higher "throughput" and potential for automation. To evaluate this assay we have compared sensitivity of aequorin in detecting receptor-mediated Ca^{2+} transients with measurements of Ca^{2+}-activated Cl current ($I_{Cl}(Ca)$) under voltage-clamp. In this study $I_{Cl}(Ca)$ and aequorin luminescence were measured simultaneously following challenge of receptors coupled to IP_3/Ca^{2+} or following direct injection of $CaCl_2$ into oocyte. Results of these study indicate that sensitivity of aequorin luminescence and $I_{Cl}(Ca)$ to Ca^{2+} are comparable. Both signals, however, may differ due to differential spatial distribution of intracellular Ca^{2+}: localized, sub-membrane Ca^{2+} transients, seen as brief $I_{Cl}(Ca)$ spikes, may not be detected by aequorin, which probably resides predominantly in the bulk of the cytoplasm; only Ca^{2+} elevation spreading throughout the oocyte (regenerative Ca^{2+} wave) will be seen in both assays.

Th-Pos482

AN EARLY TRANSIENT CURRENT ACTIVATES THE SWELLING-INDUCED CHLORIDE CONDUCTANCE IN CARDIAC MYOCYTES

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Hypoosmotic swelling of cultured chick cardiac myocytes induced a Ca_o^{2+}-dependent transient increase in $[Ca^{2+}]_i$ (Smith et al; *Circulation* 86(4):I-480, 1992). Studies with perforated patch clamp demonstrated a transient current (I_{swell}) associated with this swelling-induced Ca_i^{2+} transient (Hall et al; *Proc. XXXII IUPS* p.194, 1993). Conventional whole-cell patch clamp revealed that hypoosmotic cell swelling activated the same rapid transient I_{swell}, followed by a second, sustained current phase, I_{Cl}, within 2-3 min (Zhang et al; *Biophys. J.* 61:A441, 1992). This study examines the connection between I_{swell} and I_{Cl}. When the swelling-induced Ca_i^{2+} transient was buffered by incubating cells with 5mM BAPTA-AM (n=4, perforated patch clamp) or including 10mM BAPTA in the pipette filling solution (n=4, ruptured patch clamp), both I_{swell} and I_{Cl} were abolished and cells failed to undergo volume regulatory decrease (RVD). I_{Cl} was also abolished in the absence of Ca_o^{2+}, although I_{swell} usually remained (n=6/8). Ca^{2+} channel blockers, nifedipine (10μM) and verapamil (20μM), had no effect on either I_{swell} or I_{Cl} (n=6). However, the stretch-activated channel blocker, gadolinium (30μM), completely inhibited both I_{swell} (n=3) and I_{Cl} (n=5), and the RVD was compromised (n=3). These results suggest that cell swelling opens stretch-activated channels which are permeable to Ca^{2+} and possibly other cations, and Ca^{2+} influx through these channels (I_{swell}) mediates activation of the swelling-induced I_{Cl}.

Supported by NIH HL27105; AHA NC91F3 and The Walter P. Inman Fund.

Th-Pos483

WHOLE-CELL MEMBRANE CURRENTS IN CULTURED SINGLE ENDOCARDIAL ENDOTHELIAL CELLS OF THE PORCINE HEART.

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Electrophysiological properties of endocardial endothelial cells (EEC) are largely unknown. As EEC membrane properties may be important in the modulation of cardiac performance, we investigated the basic electrophysiological characteristics of EEC by applying the whole-cell mode of the patch-clamp technique to cultured EEC from the porcine right ventricle. At potentials more negative than the equilibrium potential for K-ions (E_K = -88 mV), an inwardly rectifying K-current (i_{K1}) was demonstrated. This current was inhibited by Ba- and Cs-ions (1 mmol/l), was sensitive to variations in extracellular K-concentrations ($[K]_{out}$) and similar to I_{K1} in vascular endothelial cells. At potentials more positive than E_K, EEC displayed an outwardly rectifying Cl-current. This current was activated upon transition to the whole-cell mode when ATP (5 mmol/l) was present in the pipette solution. The current was inhibited by the Cl-channel blockers DIDS (100 to 300 μmol/l) and flufenamic acid (50 to 100 μmol/l), was insensitive to $[K]_{out}$- but sensitive to $[Cl]_{out}$-variations. The current was reversibly inhibited in the presence of caffeine (5 to 10 mmol/l; 10 mmol/l EGTA in the pipette), suggesting a role of cAMP in the current activation. These results suggest that the main outwardly directed current in EEC is an outwardly rectifying Cl-current. Hence, EEC differ from most vascular endothelial cell preparations, in which the main outwardly directed current is a Ca-activated K-current.

Th-Pos484

A FIBER-OPTIC STIMULATOR FOR STUDIES OF THERMOSENSORY TRANSDUCTION.

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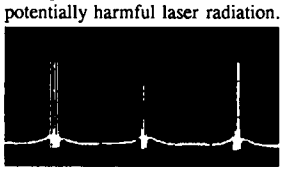
A miniature heat stimulator with temperature feed-back was built to study the thermal response properties of cultured somatosensory neurons. The stimulator consists of a 970 nm semiconductor laser (1 W max. output) and an optic fiber terminated by a miniature black-body radiator (75 μm diameter). The black-body is a quartz fiber drawn to a fine tip and coated with silicon carbide and potassium silicate. The laser is powered by a current-pulse generator whose output (0 to 2 A) is amplitude-modulated in accordance with a command signal from an arbitrary wave-form generator and temperature feed-back from a fiber-optic thermometer (Luxtron 755HG). The heat stimulator covers the entire range of temperatures (from ambient to 60°C) over which mammalian thermoreceptors and nociceptors are known to respond *in vivo*. It can stimulate single cells without exposing them to potentially harmful laser radiation.



Illustrated is a whole-cell, current-clamp recording of membrane potential of a cultured rabbit trigeminal ganglion neuron responding to three 5 sec cycles of triangular temperature variation (29 to 45°C). Supported by NSF IBN 92-11545.

Th-Pos485

EFFECTS OF INTERNAL MAGNESIUM CONCENTRATION ON DELAYED RECTIFIER POTASSIUM CURRENT IN GUINEA PIG VENTRICULAR ISOLATED MYOCYTES.

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In heart, several ion channels are sensitive to the concentration of the magnesium present at the intracellular face of the membrane. In particular the role of the (Mg_i^{2+}) ions as open channel blockers producing inward rectification is now well known in different potassium channels: I_{K1}, I_{KAch}, and I_{KATP}. Whereas effects of (Mg_i^{2+}) on delayed rectifier potassium current (I_K) have been reported, underlying mechanisms are not known. We studied the effect of changing internal magnesium concentrations using patch clamp technique in whole cell configuration and internal perfusion on guinea pig ventricular myocytes. The increase of Mg_i concentration from 0.3 mM to 3 mM induced a decrease of the outward current (I_K) elicited by a depolarizing pulse from -50 mV to +40 mV during 2 s. Determination of the voltage dependency showed that this decrease is statistically significant for positive depolarizing pulses (> +0 mV). Comparison of I_K activation curves obtained in both conditions of internal magnesium also showed a significant decrease of the amplitude values of the tail current for positive potentials (> +0 mV). Normalized activation curves could be fitted by two Boltzman equations. Switching from 0.3 mM to 3 mM (Mg_i^{2+}) induced a reduction of the amplitude of the second component without any change of the slopes. According to our results, it seems that the decrease of the outward current (I_K) when the internal concentration of magnesium is increased could be mainly due to a decrease of the slow component (I_{Ks}).

This work was supported by Conseil Regional du CENTRE (FRANCE)