was to establish a model to characterize α_{1D} Ca current using the novel technology of RNA interference.
Methods and Results: Rat neonatal cardiomyocytes (RNC) were transfected with α_{1C} specific siRNA using lipofectamine which resulted in 50% silencing at the mRNA/protein level. Limited by the low transfection effieciency (50%) in the RNC, we cotransfected α_{1C} siRNA with cy3 labeled Human GAPDH siRNA and sorted out the fluorescent cells using FACS to separate tranfected and non-transfected cells. The level of silencing of α_{1C} in enriched transfected cells reached only 65% efficiency. To achieve higher transfection efficiency, we generated and infected RNC with a lentivirus construct carrying the α_{1C} siRNA sequence under a U6 promoter. Using this model, we achieved 100% transfection efficiency, and more than 90% silencing of the α_{1C} gene confirmed by real-time PCR, Western blot, and immunoflourecence. These biochemical results were confirmed electrophysiologically by measurements of total L-type Ca current which was reduced by 80% in transfected cells.
Conclusion: Lentiviral shRNA is an efficient model for post-transcriptional gene silencing of ion channels in primary cardiomyocytes. This novel approach provides a valuable mean for assessing the differential roles of α_{1C} and α_{1D} Ca channels in native cardiomyocytes and could be used to examine their roles in physiological and pathological settings.

935-Pos Board B814

Construction Of Functional N-type Ca2+ Channels (Cav2.2) With Accessible External Epitope Tags Suitable For Live Cell Labeling

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Recent studies of voltage-gated Ca^{2+} channels indicate that mechanisms other than alterations in gating contribute to modulation. For example, internalization of receptor/channel complexes (e.g., ORL1) or formation of non-conducting species (e.g., RGK proteins), potentially contribute to $Ca_V2.2$ function. An essential technique for investigating these phenomena is the ability to specifically label fully functional $Ca_V2.2$ α-subunits (*CACNA1B*) in the plasma membrane of living cells. Towards this end, we attempted a systematic insertion of a hemagglutinin (HA) epitope tag into each of the 12 predicted extracellular loops of the rat $Ca_V2.2$ α-subunit. The first round of mutagenesis resulted in the addition of sites to 1E1 (domain 1, extracellular loop 1), 1E2, 2E2, 3E1, 3E2 and 4E3. A sequence coding for a short flexible linker plus two contiguous copies of the hemagglutinin epitope was ligated into each site. The resulting constructs were electroporated into HEK293 cells along with plasmids encoding Ca^{2+} channel β_{2a} and $\alpha_2\delta$ subunits, and the K^+ channel IRK1. Robust ''rim type'' immunofluorescent labeling (in living cells) was detected for 4 of 6 clones (1E2, 3E1, 3E2 and 4E3). Expression of these constructs in HEK293 cells produced channels with electrophysiological properties similar to wildtype as determined by whole-cell patch-clamp with 10 mM Ca^{2+} as the charge carrier. Average peak currents were (in nA) -5.4 ± 0.7 for the control and -3.8 ± 0.7, -1.2 ± 0.2, -3.1 ± 0.7 and -4.0 ± 0.7 for 1E2, 3E1, 3E2 and 4E3, respectively. The IV curves and individual current trajectories for each clone were superficially similar to the wildtype. These clones should provide powerful tools for the study of trafficking and modulatory mechanisms of $Ca_V2.2$. Additionally, the tagging strategy may be applicable to additional members of the Ca_V and Na_V families of voltage-gated channels.

936-Pos Board B815

Role Of Gamma Subunit In The Targeting Of Functional Cardiac L-Type Ca^{2+} Channels

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The cardiac isoform γ6 of the Ca^{2+} channel γ subunit family has been proposed to inhibit T-type Ca^{2+} channels by reducing the channel's availability for activation (Hansen et al., 2004, J Mol Cell Cardiol). Here we investigated how the γ6 subunit (long transcript) regulates L-type Ca^{2+} channels expressed in tsA201 cells.
First, the sub-cellular localization of the N-terminally GFP-tagged γ6 was visualized by confocal imaging. Expressed alone or with any combination of other types of subunits, the γ6 targeted well to the plasma membrane.
Second, when the γ6 subunit (long transcript) was co-expressed with α1C and β2a subunits, it dramatically reduced the density of ionic currents recorded with 10 mM Ba^{2+} from 142 ± 26 pA/pF (n=4) in control to 3 ± 2 pA/pF (n=7). The gating charge was also nearly eliminated in the presence of the γ6 subunit (from 47 ± 12 fC/pF in control to less than 5 fC/pF in cells with the γ6). The reduction in current and gating charge was independent on GFP-tagging of the γ6.
Taken together, these results indicate that the cardiac γ6 subunit regulates expression/functional targeting of cardiac L-type Ca^{2+} channels.

937-Pos Board B816

Calreticulin Negatively Regulates the Surface Expression of α1D L-Type Calcium Channel

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Background: Quality control of several proteins is strictly regulated by molecular chaperones in the endoplasmic reticulum (ER). Calreticulin, an ER Ca2+ binding chaperone, has been shown to regulate the surface expression of several membrane proteins including the cyctic fibrosis transmembrane conductance regulator (CFTR) which under oxidative stress results in its internalization and proteasomal degredation. Decrease of L-type Ca current and channel protein has been described in autoimmune associated congenital heart block. Here, we demonstrated a novel mechanism of down-regulation of α_{1D} L-type Ca channel using native human fetal cardiac cells and tsA201 cell line.
Methods and Results: Using Confocal microscopy, we found surface staining of calreticulin on cultured human fetal cardiomyocytes (HFC) gestational age 18-24 weeks. Coimmunoprecipitation from HFC using anti-α_{1D} antibody, and probing with anti-calreticulin antibody revealed a 46 kDa band corresponding to calreticulin. Overexpressing calreticulin in human embryonic kidney cells (tsA201) resulted in a decrease in surface expression of α_{1D} L-type Ca Channel. Electrophysiological studies showed that co-transfection of calreticulin with α_{1D} L-type Ca Channel led to 55% inhibition of the α_{1D} Ca current expressed in tsA201 cells.
Conclusions: These results show the first evidence that calreticulin: 1) is found on the cell surface of human fetal cardiomyocytes; 2) is coimmunoprecipitated with α_{1D} L-type Ca Channel; 3) negatively regulates α_{1D} surface expression; 4) decreases α_{1D} Ca current in tsA201 cells co-expressed with α_{1D} and calreticulin. The data demonstrated a novel mechanism of modulation of α_{1D} Ca channel, which may be involved in numerous pathological settings such as congenital heart block.

938-Pos Board B817

Inhibition Of Recycling Endosomes By Brefeldin-A Prevents KLHL1-mediated Upregulation Of α1h T-type Currents

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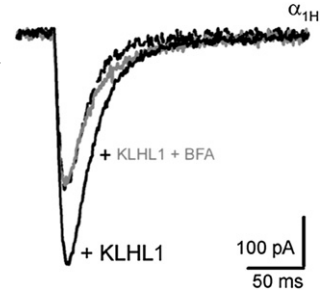
The Kelch-like 1 (KLHL1) actin binding protein interacts with α_{1H} calcium channels and increases calcium current density *via* interaction with the actin cytoskeleton, resulting in an increase in the number of channels at the membrane. We probed the effect of Brefeldin A (BFA), which affects protein transport *via* disruption of the trans-Golgi network and by blocking vesicle recycling. HEK 293 cells stably transfected with α_{1H} were incubated with various BFA concentrations; overnight incubations resulted in the elimination of basal α_{1H} currents (IC_{50}=21μM), consistent with impaired channel trafficking from the Golgi complex. Interestingly, low [BFA] (107 nM) and/or short-term incubations (3.6 μM for 1 hr) did not alter endogenous α_{1H} levels, yet completely eliminated the KLHL1 effect. Current deactivation kinetics changes originally observed in the presence of KLHL1 persisted in the presence of BFA, indicating that BFA does not affect the direct interaction of KLHL1 with α_{1H} already present at the plasma membrane.



Our data suggests KLHL1 up-regulates channel number by increasing α_{1H} re-insertion tino the membrane *via* recycling endosomes, a process that involves stabilization of the actin cytoskeleton. Supported by AHA-0615508Z (KA) and NSF- 0641141 (EPR).

939-Pos Board B818

Cardiac L-type Ca Channel as an Oxygen Sensor; Possible Involvement of Ca/Calmodulin Binding Domain

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Cellular oxygen sensing is defined as the ability of a cell to respond to changes in extracellular oxygen pressure through regulation of membrane ionic currents, mitochondrial oxidative phosphorylation or transcription of regulatory proteins. In the heart hypoxia has been shown to alter ionic currents through mitchondrial redox regulation and phosphorylation by kinases. Here we report

a novel oxygen sensing mechanism of cardiac L-type Ca channels which is independent of mitochondrial ROS and is partially regulated by PKA phosphorylation in the left ventricle. When oxygen pressure was locally decreased from 150 to 5 mmHg within 50 ms, an immediate suppression (25%) occurred in baseline I_{ca} that maximized in 40-50 seconds. This response was inhibited by PKA phosphorylation on the left but not the right ventricle. Inhibiting Ca dependent inactivation using Ba^{2+} as the charge carrier, lead to 40% suppression of I_{Ba} within the first 5-15s of exposure. This effect was independent of PKA phosphorylation and equally affected both ventricles. Inhibiting SR Ca release with 5uM thapsigargin did not mimic the response seen with Ba^{2+}. However, inhibiting Calmodulin using CaM inhibitory peptide 290-309 partially suppressed phosphorylated I_{ca} in the left ventricle. This effect was also present in HEK 293 cells expressing all subunits of the recombinant L-type Ca channel. Furthermore, mutating 80 amino acids in the Ca binding/IQ domain of the alpha 1C subunit which removes Ca dependent inactivation and leads to similar kinetics of I_{Ba} and I_{Ca}, abolished the suppression of I_{Ba} under low O_2. Based on these observations we propose that Cardiac L-type channels have oxygen sensing properties and that Ca/Calmodulin binding domain is a key site in this process.

940-Pos Board B819

Calcium channels regulate myocardial compaction

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BACKGROUND: Calcium regulation is important for cardiac myocyte function and cardiac development. For example, calcium channel blockade and deletion of calcium-regulatory proteins leads to abnormal cardiac morphogenesis. We have previously demonstrated that deletion of the major calcium channel isoform (CaV1.2) from the developing heart leads to grossly normal cardiac structure, but late embryonic demise.

OBJECTIVE: Determine the effects of calcium channel deletion on cardiac development.

METHODS: Global deletion of the major cardiac LTCC isoform, CaV1.2, was obtained using floxed-CaV1.2 mice (Dr. Franz Hofmann) mated to beta-actin-Cre mice. Conditional deletion of CaV1.2 in the AHF was obtained using Mef2c-AHF-Cre mice (Dr. Brian Black). Specimens at various stages of gestation were examined by embryonic echocardiography and cardiac heart rates and left and right ventricular shortening fractions were quantified. Embryos were harvested and examined for changes in gross morphology and cardiac morphology by visual observation and histological methods. Compaction of the ventricular myocardium was quantified on histologic sections.

RESULTS: Embryos containing global or conditional deletions of CaV1.2 die at E14 and E15, respectively. Approximately 1 day prior death, null embryos had abnormal cardiac function with depressed shortening fraction and abnormal heart rates. Over the next day, cardiac failure became apparent, with evidence of pericardial effusions and body wall edema. Approximately 2 days prior to death, the ventricular myocardium appeared to lack compaction of the trabeculae into the compact layer of the myocardium. Prior to this, the embryonic hearts appeared normal.

CONCLUSIONS: The presence of normal calcium channels is important for late maturation of the embryonic ventricular myocardium. Deletion of CaV1.2 caused ventricular non-compaction followed by depressed cardiac function, heart failure, and late embryonic demise.

941-Pos Board B820

Enhancement of the Cav3.1 Channel Activity by PKA in Ventricular Myocytes of a1G Transgenic Mice

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Low voltage-activated T-type Ca^{2+} channels (Cav3 or TTCC) play an important role in regulating the pacemaker activities in the heart. Since adrenergic system is critical for heart rate regulation and the TTCC is involved in cardiac rhythmn generation, it is important to examine the regulation of the TTCC by the adrenergic-PKA system. In this study, we sought to resolve the question whether Cav3.1 in cardiac myocytes is regulated by PKA. **Methods:** Cav3.1 α1G transgenic mice were established with the cardiac specific and inducible system engineered by the Robbins group. Whole cell voltage clamp was used to measure the $I_{Ca\text{-}T}$ before and after isoproterenol application. $I_{Ca\text{-}T}$ was also recorded with or without cAMP (10μM) in the pipet. **Results**: (1) There is robust $I_{Ca\text{-}T}$ (25.3 ± 12.5pA/pF, n=13) in ventricular myocytes isolated from α1G TG mice but no $I_{Ca\text{-}T}$ was observed in ventricular myocytes from control mice; (2) $I_{Ca\text{-}T}$ in α1G TG myocytes was significantly increased by isoproterenol application (before vs. after: 15.0 ± 3.3pA/pF, n=4 vs. 11.7 ± 4.7pA/pF, n=4, at -40mV, $p<0.05$). This indicates Cav3.1 channel activity was probably up-regulated by isoproterenol-activated PKA in myocytes isolated from adult α1G transgenic mice. (3) cAMP can greatly increase both T-type (with vs. without cAMP: 48.9 ± 29.2pA/pF, n=4 vs. 25.3 ± 12.5pA/pF, n=13, maximal $I_{Ca\text{-}T}$, $p<0.05$) and L- type calcium currents (with vs. without cAMP: 19.9 ± 7.4pA/pF, n=4 vs. 7.6 ± 3.7pA/pF, n=13, maximal $I_{Ca\text{-}L}$, $p<0.05$). This further confirms the up-regulation effect of PKA on the Cav3.1 channel activity**. Conclusions:** For the first time, we found that PKA activation enhances Cav3.1 channel activity in ventricular myocytes of mice. This finding may shed a light on the physiological and pathophysiological (arrhythmogenic) effects of sympathetic regulation of pacemaker activities in the heart through T-type calcium channels.

942-Pos Board B821

Cardiovascular profile of newly developed Diltiazem analogs

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Many diltiazem related L-VDCC blockers were developed using a multidisciplinary approach. This current study was to investigate and compare diltiazem with to the newly developed compounds by mouse Langendorff-perfused heart, Ca^{2+}-transients and on recombinant L-VDCC. Five particular compounds were selected by the ligand-based virtual screening procedure (LBVS) (5B, M2, M7, M8 and P1). Wild-type human heart and rabbit lung α_1 subunits were expressed (combined with the regulatory $\alpha_2\delta$ and β_3 subunits) in *Xenopus leavis* oocytes using a two-electrode voltage clamp technique. Diltiazem is a benzothiazepine Ca^{2+} channel blocker used clinically for its antihypertensive and antiarrhythmic effects. Previous radioligand binding assays revealed a complex interaction with the benzothiazepine binding sites for M2, M7 and M8. (*Carosati E. et al. J. Med Chem. 2006, 49; 5206*). In agreement with this, the relative order of increased rates of contraction and relaxation at lower concentrations ($<10^{-6}$M) in un-paced hearts was M7>M2>M8>P1. Similar increases in Ca^{2+} transients were observed in cardiomyocytes. Diltiazem showed negative inotropic effects whereas 5B had no significant effect. Diltiazem blocks Ca^{2+} currents in a use-dependent manner and facilitates the channel by accelerating the inactivation and decelerating the recovery from inactivation. In contrast to diltiazem, the new analogs had no pronounced use-dependence. Application of 100 μM M8 and M2 showed ~10% tonic block, shifted the steady-state inactivation in hyperpolarized direction and the current inactivation time was significantly decreased compared with control (219.6 ± 11.5 ms, 226 ± 14.5 vs. 269 ± 12.9 ms). Contrary to diltiazem, the recovery from the block by M8 and M2 was comparable to control. All of the compounds displayed the same sensitivity on the Ca^{2+} channel rabbit lung α_1 except P1. Taken together, these findings suggest that M8 and M2 might directly decrease the binding affinity or allow more rapid dissociation from the benzothiazepine binding site.

943-Pos Board B822

Charge-dependent And Isoform-specific Interactions Between ProTxii And T-type Calcium Channels

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ProTxII, peptide toxin isolated from the venom of the tarantula Thrixopelma puriens, modifies voltage-dependent activation of both T-type calcium (Ca) channels and voltage-gated sodium (Na) channels. In the presence of ProTxII (5μM) the voltage at half maximal activation (V1/2) of the CaV3.1 isoform is shifted positive (>25mV) and maximum conductance (Gmax) decreases (~50%). Interestingly, the toxin's effects on this channel were completely precluded in the presence of high extracellular divalent concentrations indicating a role for surface charge-like, electrostatic interactions with the channel. Several mutant toxins in which individual basic residues were neutralized were tested for activity on CaV3.1. Three of these mutants, R13Q, R22A, and K28A, significantly disrupted the ability of the toxin to both shift channel activation and decrease Gmax. Two other mutations: K4Q and K14A, showed minimal or no effect, thus indicating an important yet specific role of charge in ProTxII's interaction with CaV3.1. The gating kinetics of T-type Ca channels varies among the three known isoforms suggesting there might be differences in the gating structures and, therefore, potential gating modifier toxin interaction surfaces as well. In CaV3.3, 5μM ProTxII reduced Gmax by approximately 60%, similar to what was seen for CaV3.1. However, unlike in CaV3.1, this concentration of toxin produced only a minimal shift in voltage dependent activation (2mV). These results suggest significant differences in the extracellular surface of T-type Ca channels across isoforms, particularly in terms of surface charge distribution close to one or more of the channels' voltage sensors.

944-Pos Board B823

Modeling L-type Calcium Channel with Dihydropyridines

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