channels, whose primary contribution is to the upstroke and plateau of the action potential; Nav1.5 [I_{Na}] (heterologously expressed) and the native cardiac L-type Ca channel, Cav1.2 [I_{CaL}] (cardiac myocytes). The biophysical properties of these two cardiac ion channels have been extensively characterized and each ion channel assay has been pharmacologically validated with reference compounds. In conclusion, this approach is part of our continuing effort to move to a cellular based *in vitro* safety approach to provide mechanistic SAR for solving cardiac safety issues. To date, our ion channel toolbox includes: hERG, Kir2.1, KvLQT1/minK, Nav1.5 and the native cardiac L-type Ca channel (Cav1.2); to be used on an as needed basis for cardiac safety evaluation.

1343-Pos Board B187

Do hERG Enhancers and Blockers Compete?

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HERG (human ether a go-go related gene) encodes a cardiac potassium channel that has been linked to delayed repolarization. Due to the large vestibule of the hERG channel pore, many structurally dissimilar compounds are able to block the hERG channel. This, along with specific requirement of hERG data by regulatory authorities, has added to the difficulty of drug discovery. Recently, we have discovered a series of compounds (hACTs) that do not block hERG, but actually enhance hERG current. hACT-1({4-[4-(5-trifluoromethyl-1H-pyrazol-3-yl)-phenyl]-cyclohexyl}-acetic acid) enhanced hERG current by 50 % at 60 μM. In addition, hACT-1 caused concentration-dependent shortening of the action potential duration in canine Purkinje fibers and guinea pig atrial tissue. Preliminary studies suggest that binding of hACT-1 (60 μM) does not overlap sites of typical hERG blockers. hACT-1 did not displace radio labeled dofetilide. Also, in whole-cell voltage clamp studies, combination of hACT-1 with known hERG blockers (i.e., sotalol and terfenadine) suggest that the compounds are not competing for the same binding site. When applied simultaneously with a hERG blocker, the onset of hERG enhancement with hACT-1 occurs prior to block with either sotalol or terfenadine. Block with sotalol (150 μM) occurs at the same magnitude when used alone (42 %), or in combination with hACT-1 (44 %). Similarly, the enhancement of hERG current by hACT-1 is independent of sotalol block, just as the block of hERG current by sotalol is independent of hACT-1 current enhancement. These effects demonstrate that the binding site for enhanced hERG current is different than the binding site for block.

1344-Pos Board B188

A Novel *SCN5A* Mutation Associated With Brugada Type ECG And Intraventricular Conduction Defects

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Background: Mutations of SCN5A, gene encoding α-subunit of cardiac sodium channel, can cause mixed phenotypes of Brugada syndrome (BrS) and cardiac conduction diseases (CCDs).
Methods: We have identified a novel nucleotide change of *SCN5A* (4178T>G) which results in a nonsense mutation, L1393X, in a 36 year-old Caucasian male who presented with intraventricular conduction delays and BrS type ECG change. To study biophysical characteristics of L1393X-SCN5A, electrophysiological and immunostaining studies were performed using mammalian expression systems.
Results: While WT-SCN5A showed significant currents (93.3 ± 10.6 pA/pF; 1 μg plasmid), L1393X (5 μg) did not generate any significant currents in NIH-3T3 cells. The cells co-transfected with WT (0.5 μg) and L1393X (0.5 μg) showed approximately 50% current amplitudes compared to the WT (1 μg). Voltage-dependency of the steady-state activation and inactivation was not affected by the co-transfection of L1393X. Immunohistochemical stainings demonstrated that L1393X proteins were expressed in the plasma membranes.
Conclusion: Our study demonstrated that L1393X-SCN5A does not form functional channel proteins, which might account for the patient's mixed phenotypes of BrS and CCDs.

1345-Pos Board B189

Effects Of Silencing Synapse Associated Protein-97 On Cardiac Potassium Currents

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Introduction: Synapse associated protein-97 (SAP97) is a scaffolding protein expressed in cardiac myocytes. Previous studies have suggested that SAP97 interacts with and modifies properties of ion channels. We have investigated the functional effect of silencing SAP97 on major repolarizing currents in adult rat ventricular myocytes (ARVMs). **Methods:** SAP97 was silenced using a shRNA expressing adenovirus. The standard patch clamp technique was used to investigate the effects of this silencing on potassium currents in ARVMs. Control experiments were carried out in ARVMs infected with a GFP expressing adenovirus. **Results:** Western blot analysis showed that SAP97 was silenced in ARVM after 3 days in culture. In SAP97 silenced ARVMs, I_{K1} density was reduced by ~50% when measured at -100 mV (Holding potential (HP) = -50 mV). Average current density was -1.85 ± 0.3 pA/pF, n=12 as compared to -3.76 ± 0.5 pA/pF, n = 6 in control cells. Depolarization-activated (at +60 mV, HP = -70 mV) currents in the ARVM were fitted with a two-exponential function for analysis. Amplitude and kinetic analysis of the fits showed that there was a 30% decrease in the current density of the first component in SAP97 silenced ARVMs (9 ± 1.9 pA/pF, n=5) as compared to control (13.5 ± 0.9 pA/pF, n=4). SAP97 silencing however did not significantly change the kinetics of the first component. Time constants averaged 68 ± 12 msec and 1.1 ± 0.1 sec in control versus 121 ± 24 msec and 1.47 ± 0.21 sec in SAP97 silenced cells. Compared to the control cells, there was no change in the current density of the second component in SAP97 silenced cells. Current amplitude averaged 3.1 ± 0.4 pA/pF (n=5) and 3.7 ± 0.2 pA/pF (n=4), respectively for SAP97 silenced ARVMs and control. These results suggest that the silencing of SAP97 has differential effects on potassium currents in adult cardiac myocytes.

1346-Pos Board B190

Evidence And Functional Impact Of A New K+ Channel In Mouse Ventricular Fibroblasts

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In the heart, fibroblasts represent the major cell type. They contribute to the production of the extracellular matrix. Cardiac remodelling during pathological injury is associated with differenciation of fibroblasts into myofibroblasts. The aim of this study was to characterize at molecular and functional levels a new K conductance in these cells.
Among K channel transcripts which were screened by high-throughput real-time PCR, SUR2 and Kir6.1 mRNAs were found to be the most abundant. Western-blots showed that SUR2 and Kir6.1 protein expression levels increased with culture duration as fibroblasts differenciated into myofibroblasts. In the inside-out configuration of the patch-clamp technique, SUR2/Kir6.1 K channels were recorded and showed insensitivity to ATP, inhibition by glibenclamide and activation by pinacidil and UDP. These properties are similar to those reported by Yamada et al (1998) for the SUR2/Kir6.1 molecular signature. In the whole cell configuration, these channels gave rise to a macroscopic glibenclamide-sensitive current which was activated by pinacidil and which amplitude increased with culture duration. This current was also activated by the endogenous sphingolipid sphingosine-1-phosphate (S1P) at the nM concentration range. The activation of this current was found to stimulate cell proliferation and to decrease IL-6 secretion. All these functional effects occurred for culture duration greater than 5 days.
In conclusion this work shows for the first time the presence of a glibenclamide-sensitive current which appears during differenciation of fibroblasts into myofibroblasts. This SUR2/Kir6.1 current, which may be activated in pathological conditions where fibroblasts differentiate into myofibroblasts and where S1P level increases, may modulate cardiac ventricular function.

TRP Channels

1347-Pos Board B191

The TRP Domain Of TRPC3 Is Essential But Not Sufficient For Erythropoietin- Regulated Activation Of TRPC3

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TRPC3 and TRPC6 are nonselective calcium channels and two members of the canonical transient receptor potential (TRPC) subfamily expressed on human erythroblasts. Although they are 73% identical in their amino acids sequence, they respond differently to erythropoietin (Epo) stimulation. Epo stimulates a significantly greater increase in calcium influx through TRPC3 (236 ± 7% increase above baseline) compared to TRPC6 (74 ± 5% above baseline). TRPC6 also inhibits Epo-stimulated calcium influx in cells cotransfected

with TRPC3 and TRPC6. We observed differential expression of these two channels during development of primary erythroid progenitors. We also showed with immunoprecipitation that endogenous TRPC3 and TRPC6 interact in the erythroid cell line TF-I. The increasing TRPC3/TRPC6 ratio during differentiation of erythroid cells correlated with increased Epo-stimulated calcium influx. We investigated the identity of the domains involved in Epo-stimulated TRPC3 activation and determined that the TRPC3 carboxyl terminus (C-domain) is required and sufficient for TRPC3 response to Epo. Furthermore, substitution of the TRPC3 TRP domain with that of TRPC6 eliminated the Epo-stimulated rise in $[Ca^{2+}]_I$, but substitution of TRPC6 TRP domain with that of TRPC3 did not reconstitute activity. In summary, our observations indicate that the TRPC3/TRPC6 ratio is physiologically relevant, suggesting that TRPC6 plays an important role in the proliferation and differentiation of erythroid cells through its role in modulating Epo-stimulated activation of TRPC3. In addition, the TRPC3 TRP-domain is critical in TRPC3 activation by Epo.

1348-Pos Board B192

Cellular Targeting And Function Of Trpc4 Channels In Human Vascular Endothelium

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TRPC4 has been suggested as a Ca^{2+} entry channel, which governs endothelial permeability. In an attempt to identify mechanisms that link TRPC4 function and cell adhesion, we tested the hypothesis that TRPC4 is part of the local signal transduction machinery within adherens junctions. In HEK293 cells transiently co-transfected with VE-Cadherin and TRPC4 constructs, we observed a co-localization of the two proteins within cell-cell contacts. In human microvascular endothelial cells (HMEC), endogenous TRPC4 was found to co-precipitate with two essential components of junctional complexes, VE-cadherin and β-Catenin. Membrane presentation of TRPC4 strongly promoted the formation of cell-cell contact and modified the response to pro-inflammatory stimuli. We observed that both basal- as well as agonist-stimulated Ca^{2+} influx were substantially augmented by the formation of cell-cell contact in HMEC. Furthermore, we found a significant increase in TRPC4-mediated Ca^{2+} signals and membrane currents in response to the formation of cell-cell contacts in TRPC4 and VE-cadherin-expressing HEK293 cells. We propose recruitment of TRPC4 proteins into cell-cell contacts as a key mechanism for control of endothelial Ca^{2+} signalling.

1349-Pos Board B193

Amino Acid Residues Within The Putative Pore Region Of TRPC3 As Determinants Of Channel Regulation

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TRPC3 channels are typically activated in response to stimulation of PLC-coupled receptors and are considered to play a role in a variety of tissues. So far little information is available on structural determinants of channel function. In this study we set out to modify putative permeation-relevant residues in this ion channel by mutagenesis. The impact of the mutations on TRPC3 function was characterized in HEK293 cells by patch-clamp experiments as well as calcium imaging. A triple mutation (E630A, D639A, E644A) within the putative pore region resulted in enhanced basal activity and in a more linear IV-relation. Substitution of charge polarity at these positions (E→Q, D→N) failed to induce detectable changes in PLC-dependent activation, rectification or selectivity. Similarly exchange of a single negative residue in this region (D639A) failed to affect channel function as well. Surprisingly, double substitution of E to Q near the putative external vestibule (residues 615 & 616) generated a TRPC3 channel that no longer responds to PLC-mediated stimulation, while substitution of a single charged residue (E616) did not induce functional consequences. Furthermore, we tested for the localisation of particular regions of the protein in the outer vestibule and/or the permeation pathway by a cysteine scanning strategy.

In summary, we identified critical amino acid residues within the putative pore region which may be important determinants of channel regulation and/or gating.

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1350-Pos Board B194

Trpc3 Encodes Native Constitutively-active Cation Channels Controlling The Resting Membrane Potential In Airway Smooth Muscle Cells

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Native constitutively-active cation channels have been proposed to play an important role in physiological and pathological cellular responses in a variety of cells. In the present study, we aimed at determining the molecular identity and functional role of native constitutively active cation channels remain in smooth muscle cells (SMCs). Using Western blot analysis, we have shown that TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6 proteins were expressed in airway SMCs. Single channel recordings indicate that anti-TRPC3 antibodies blocked the activity of constitutively-active cation channels, while anti-TRPC1, TRPC4, TRPC5 and TRPC6 antibodies had no effect. Anti-TRPC3 antibodies, but not anti-TRPC1, TRPC4, TRPC5 and TRPC6 antibodies, significantly hyperpolarized the resting membrane potential. Similarly, siRNA-mediated TRPC3 gene knockdown greatly diminished the constitutively-active cation channel activity and hyperpolarized the resting membrane potential, whereas TRPC1 and TRPC6 gene knockdown did not affect either the channel activity or the resting membrane potential. Intriguingly, we have also found that in asthmatic Airway SMCs, the activity of constitutively-active cation channels was significantly augmented, the resting membrane potential was depolarized, and TRPC3 protein expression was increased. Anti-TRPC3, but not ani-TRPC1 and TRPC6 antibodies prevented the constitutively-active cation channel activity and hyperpolarized the resting membrane potential in asthmatic airway SMCs. Taken together, these findings demonstrate that TRPC3 encodes the native constitutively-active cation channels, playing an important role in controlling the resting membrane potential in SMCs. Moreover, TRPC3-encoded channels may contribute to asthma and other smooth muscle diseases.

1351-Pos Board B195

Characterization Of A Novel TRPC6 Mutant Identified In FSGS Patients

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TRPC6 is a Ca^{2+}-permeable non-selective cation channel. Gain of function mutations of TRPC6 have been shown to cause focal segmental glomerulosclerosis (FSGS). Among six mutants of TRPC6 indentified in FSGS, three of them cause increase in Ca^{2+} influx. It appears that the enhanced Ca^{2+} influx underlies TRPC6 mutation associated FSGS. However, it is unclear how different mutations lead to gain of channel function and increase in Ca^{2+} influx. Here we report a novel TRPC6 mutant, M132T, which causes early-onset FSGS. Whole cell patch clamp experiments showed that current amplitude of M132T was 3- to 5-fold larger (476.9 ± 55.9 pA/pF) than that of wild-type (wt) TRPC6. Interestingly, while the wt TRPC6 exhibited apparent time-dependent inactivation, M132T did not show inactivation or only minor time-dependent decline of inward current. Inward Ca^{2+} current of M132T measured in 10 mM Ca^{2+} external solution was 10-fold larger than that of wt TRPC6. Moreover, Ca^{2+} influx of M132T was also significantly bigger than wt TRPC6. To understanding the mechanism of slow inactivation kinetics of M132T, we applied various intracellular Ca^{2+} concentrations and compared inactivation processes of M132T and wt TRPC6. We found that higher Ca^{2+} concentration was required to induce M132T inactivation in comparison with wt TRPC6, suggesting that M132T is less sensitive to intracellular Ca^{2+} induced inactivation. Taken together, our results indicate that the lack of inactivation may confer the enhanced Ca^{2+} influx in M132T. Further investigation is required to understand the mechanism of enhanced channel functions of TRPC6 mutants in FSGS.

1352-Pos Board B196

Pore Helix Mediates Proton Block of Vanilloid Receptors

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Tissue acidosis occurs during inflammation and injury, and modulates many receptors and ion channels on the pain pathway including the capsaicin ion channel TRPV1. Extracellular low pH exerts several effects on the function of TRPV1. Extreme acidification leads to direct activation of the channel, while mild acidic pH potentiates its response to other stimuli. Paradoxically, protons also inhibit the unitary conductance of the channel. This inhibitory effect confers TRPV1 a similar maximum response at low pH in spite of increased agonist sensitivity, thereby limiting the ion flux into cells. Proton-mediated pore block has been studied extensively in other ion channels. Two representative mechanisms have been proposed, one involving competitive inhibition with permeating ions, and the other by reducing the surface potential of membranes. We have examined these mechanisms for proton block of TRPV1. Surprisingly, we found that neither mechanism could adequately account for the full blocking effect of protons. Mutagenesis experiments revealed that, in addition to a residue at the pore entrance, another residue