inactivation, such that the salt-bridge mutants E132Q-Kir1.1b and R128Y-Kir1.1b inactivated in 100mM K solutions after a transient acidification. However 300mM external K (but not 200mM Na +100mM K) protected E132Q and R128 from inactivation during this acidification, suggesting an altered K sensitivity in these mutants. External application of a modified honey bee toxin (TPNQ) protected wild-type ROMK from inactivation in 1mM K and protected E132Q or R128Y from inactivation in 100mM K, suggesting that TPNQ binding to the outer mouth of the channel stabilizes the conducting state. Nonetheless, TPNQ was unable to protect either E132Q or R128Y from inactivation in 1mM external K. However, both E132Q and R128Y were protected from inactivation in 1mM K either by a mutation that disrupted transmembrane helix H-bonding (K61M-Kir1.1b) or by a mutation that stabilized a selectivity-filter to helix-pore linkage (V121T-Kir1.1b). Our results are consistent with an inter-intra subunit salt bridge near the outer end of the selectivity filter that stabilizes the conductive state of the channel.

2384-Pos Board B354

Gating Sensitive Residues In The Pore Of An Inwardly Rectifying Potassium (Kir) Channel

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Intracellular pH gates inwardly rectifying potassium (Kir) channels by controlling the reversible transition between the closed and open states. This gating mechanism underlies important aspects of Kir channel physiology and pathophysiology. H^+ inhibition is thought to be triggered by protonation of residues within the cytoplasmic domains. This then causes major conformational rearrangement of the TM helices and slide helix resulting in closure of the gate at the helix bundle crossing. To identify residues important for this gating process we performed a systematic alanine scan in Kir1.1 channels over the entire transmembrane pore structure of the channel (residues 61 - 192) and measured pH sensitivity of the individual mutants in inside-out patches. We identified gating sensitive residues in both TM1 and TM2 that appear to make up an intra-subunit gating interface as well as a cluster of residues in the proximal part of the slide helix extending into TM1. Two highly conserved phenylalanines (F84, F88) in TM1 seem be of particular importance as they had dramatic effects on the pH gating kinetics. Assuming that the mutations do not affect the cytoplasmic pH sensor directly, a change in IC50 therefore represents a change in the stability of the closed state relative to the open state. Intriguingly, most of the gating sensitive mutations (17 out of 19) increased the IC50 for pH inhibition (from 6.4 (wild-type) up to 8.5) indicating that the mutations had a marked tendency to disturb the stability of the open state more severely than the closed state. This suggests that the open state in Kir channels is structurally more optimised than the closed state.

2385-Pos Board B355

State-dependent Cysteine Modification during pH and PIP_2 Gating in Kir Channels

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Inhibition by intracellular H^+ (pH-gating) and activation by phosphoinositides such as PIP_2 (PIP_2-gating) are key regulatory mechanisms in the physiology of inwardly-rectifying potassium (Kir) channels. Our recent findings suggest that PIP_2 gating and pH gating underlie similar conformational change at the helix bundle crossing, however, little is known about the structural changes in the cytoplasmic domains. Here we explore the state-dependent changes in accessibility of three endogenous cysteines (C175, C49, C308) in Kir1.1 channels occurring during PIP_2 and pH gating. C175 in the inner pore cavity is modified by MTSET in the open state, but protected from modification in the closed state induced by either low intracellular pH or PIP_2 depletion. This confirms the concept that the helix bundle crossing represents the gate controlled by pH and PIP_2. C49 in the N-terminus is protected from modification in the open state but can be modified in the closed state induced by either low pH or PIP_2 depletion indicating a similar conformational change in this region. C308 in the C-terminus can only be modified in the closed state induced by PIP_2 depletion but is protected in the open state and as well in the pH-inhibited closed state. A homology model of Kir1.1 shows that C308 is located in close proximity to the PIP_2 binding site indicating that PIP_2 either directly, or by a conformational change at C308 protects this residue from modification. The lack of C308 modification in the pH inhibited state suggests that pH induced channel closure occurs with PIP_2 tightly bound (thereby protecting C308), which is also consistent with our measurements on the kinetics of pH and PIP_2 induced channel activation.

2386-Pos Board B356

Role of Kir 2-caveolin-1 interactions in the sensitivity of Kir to cholesterol

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Our earlier studies have shown that Kir2 channels are strongly suppressed by the elevation of cellular cholesterol and enhanced by cholesterol depletion. We have also shown that Kir2 channels partially partition into cholesterol-rich membrane domains suggesting that interactions between the channels and other components of these domains may be critical for the regulation of the channels. It is also known that cholesterol interacts with caveolin-1, a scaffolding regulatory protein residing in these domains. In this study we test whether Kir2 channels are regulated by caveolin under different cholesterol conditions. Our data shows that Cav-1 co-immunoprecipitates with both Kir2.1 and Kir2.3 channels, suggesting that Cav -1 may be involved in the regulation of Kir2 channels. Furthermore, we show here that bone-marrow derived macrophages isolated from $Cav^{-/-}$ knock-out mice have larger Kir currents than cells isolated from control animals supporting the hypothesis that Cav-1 regulates Kir channels. Finally, we also show that sensitivity of Kir currents to cholesterol in $Cav^{-/-}$ cells is weaker than in control cells providing further evidence for the role of Cav-1 in the sensitivity of Kir channels to cholesterol.

2387-Pos Board B357

Long Qt Syndrome Mutations In Caveolin-3 Cause Loss Of The Kir2.1-mediated Inward Rectifier Potassium Current (i_{K1})

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Caveolin-3 (CAV3) is a key structural protein in cardiac caveolae that serves as an anchoring protein and a regulatory element for protein signaling in human cardiac myocytes. We have demonstrated previously that mutations in *CAV3*-encoded caveolin-3 are associated with long QT syndrome (LQT9) and increase late sodium current (I_{Na}).

We postulated that CAV3 may also regulate other ion channels such as KIR2.1, the channel responsible for the cardiac inward rectifier current I_{K1} and also the channel underlying Andersen-Tawil Syndrome (ATS1/LQT7). We therefore tested the four LQT9-associated mutations (F97C, S141R, T78M, A85T) for effects on inward rectifier channel KIR2.1. Wild-type (WT) Kir2.1 was expressed transiently in HEK293 cells with either WT stably expressed or mutant CAV3 proteins with IRES-GFP transiently expressed. Kir2.1 currents were measured using whole-cell patch clamp technique. WT CAV3 had no effect on Kir2.1 current. However, F97C-, S141R-, A85T-, and T78M-CAV3 mutations abolished both inward and outward I_{K1} current density. At -120mV inward I_{K1} current density was reduced by 59% (F97C), 55% (S141R), 80% (A85T) and 41% (T78M), $p \leq 0.02$. At -40mV outward I_{K1} current density was reduced by >96% for F97C, S141R, and T78M ($p \leq 0.04$), and was reduced by 68% by A85T ($p \leq 0.04$). This marked loss of I_{K1} function, over the physiological voltage range, important for terminal repolarization, suggests that CAV3 mutations may cause the LQT phenotype by a cumulative effect on I_{K1} and I_{Na}. More generally, it suggests that caveolin-3 is a novel Kir2.1 channel interacting protein. The detailed mechanism of this interaction and the implications for cardiac electrophysiology require further investigation.

2388-Pos Board B358

Epidermal Growth Factor Receptor Tyrosine Kinase Stimulates Human Inward Rectifier Potassium (Kir2.3) Channels

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Protein tyrosine kinases (PTKs), in addition to the mediation of cellular events such as cell growth, differentiation, etc., regulate ion channels. Although Kir2.3 channel plays a crucial role in the repolarization and membrane potential stabilization of neurons and myocardium, modulation of this channel is not fully understood. The present study investigated whether/how human Kir2.3 channel is modulated by PTKs and protein tyrosine phosphatases (PTPs) in HEK 239 cells stably expressing Kir2.3 gene using approaches of whole-cell patch voltage clamp, immunoprecipitation and Western blot, and site-directed mutagenesis. We found that epidermal growth factor (EGF, 100 ng/ml) and PTPs inhibitor orthovanadate (1 mM) significantly enhanced Kir2.3 channel current, while the broad spectrum PTKs inhibitor genistein and the selective EGF receptor kinase inhibitor AG556, but not the Src-family PTK inhibitor PP2 or the platelet-derived growth factor receptor kinase inhibitor AG1295, suppressed

the current. The inhibitory effect of Kir2.3 current by genistein or AG556 was fully countered by EGF or orthovanadate. In addition, tyrosine phosphorylation level of Kir2.3 channel was increased by EGF or orthovanadate, but decreased by genistein or AG556. The reduced phosphorylation level by genistein or AG556 was reversed by EGF or orthovanadate. Interestingly, the response of Kir2.3 channel to EGF or AG556 disappeared in Kir2.3 Y234A mutant. Our results demonstrate the novel information that human Kir2.3 channel is stimulated by EGFR tyrosine kinase via phosphorylating the channel at Tyr234.

2389-Pos Board B359

The Behavior of Ions Inside the Cytoplasmic Domain of Inward Rectifier Potassium Channels

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Inward rectifiers are a subfamily of potassium channels that control the amounts of outward K^+ current in a cell. These channels achieve rectification through intracellular block by strongly charged cations such as Mg^{2+} and polyamines like spermine (SPM^{4+}). The large cytoplasmic domain that extends the ion permeation pathway has been shown to be an important determinant of conductance and rectification, however the organization of ions within this part of the pore is not well understood. In this study, the properties of ions inside the cytoplasmic domains of a weak (Kir1.1/ROMK) and strong (Kir2.1/IRK) rectifier are investigated via explicit solvent molecular dynamics simulations in 1M KCl. Both channels concentrate K^+ ions in large amounts (local concentration $>$ 3M), with the highest densities near the protein surface. An additional concentrating region specific to Kir2.1/IRK is observed near the cytoplasmic opening. Simulations are also carried out with Mg^{2+} or SPM^{4+} inside the domain. Mg^{2+} interacts directly with pore-lining residues, resulting in a depletion of K^+ and increase in the local concentration of Cl^-. SPM^{4+} shows high density throughout the central pore and selectively depletes K^+ in the upper region of the pore closest to the transmembrane domain. Two long-lived states of SPM^{4+} are observed in Kir2.1/IRK: (i) inside the central pore in contact with residues $D259_1$, $E224_1$, $E224_2$ and $E299_2$ from two adjacent subunits, and (ii) near the cytoplasmic entrance interacting with residues D255, D259 and E224 on a single monomer. These results demonstrate a level of molecular specificity with respect to ion behavior within the cytoplasmic domains that could correspond to differences in rectification properties.

2390-Pos Board B360

Modelling and simulations of the inward-rectifying potassium channel Kir2.1

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The Kir2.x family of inward-rectifying potassium channels is responsible for the IK1 current in the human heart. This current stabilizes the resting membrane potential and shapes the final repolarization the ventricular action potential in the late phase. We built homology models for human Kir2.1, starting with the crystallographic structures of a chimera of mouse Kir3.1 and KirBac1.3 as templates. We performed full-atomistic molecular dynamics simulations starting with one of these models. Five production trajectories, totalling 100 ns, were obtained using different equilibrating conditions and initial numbers of potassium ions in the cavity. Analyses of these trajectories included diagonally-opposite carbonyl carbon measurements for the filter region and pore radius profiles. Results from these analyses gave insight to the gating and permeation processes of Kir2.1. For example, the 4 residues of Met180, located next to the cavity away from the filter region, were the major determinant of gating in the transmembrane domain. A cross-product indicator showed the opening extent of this gate. Further, to investigate the role of magnesium binding in the mechanism of inward-rectification, we calculated the Poisson-Boltzmann energy profiles for a magnesium ion in the pores of the models. This suggested possible magnesium binding sites.

2391-Pos Board B361

Mechanisms of Short-Term Desensitization of GIRK Channel Activity

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G protein-coupled receptors (GPCR) signaling is precisely timed. This can be achieved at the level of the G protein activation and/or at the receptor level. At the receptor level, classical desensitization is believed to be controlled by a decrease in receptors number in the membrane: Activated receptors are phosphorylated by GPCR-kinase (GRK), tagging them for arrestin binding. This in-turn leads to clathrin-mediated receptor endocytosis. This multisteps process is extended in time (min), and is dependent on initial GPCR phosphorylation by GRK. In contrast, effector regulation is controlled at much faster time scale (s). Here we used the G protein-coupled inwardly rectifying K^+ channels (GIRK), as an effector model for GPCR activation, to achieve real-time readout, in intact cells, of GPCR-mediated effector regulation. Upon GIRK activation, currents were desensitized within seconds. This regulation may serve as fast negative feedback for GPCR activation. Here we show that GIRK desensitization rates are dependent on GRK2 activity. GRK2 accelerates desensitization of currents induced by some $G\alpha_{i/o}$ coupled receptors, but not all. In contrast, silencing endogenous GRK2, results in a decreased in the desensitization rates. A dominant-negative mutant lacking kinase activity did not affect the ability of GRK2 to accelerate currents desensitization, suggesting the lack of GPCR classical desensitization in this process. In contrast, GRK2 mutant displaying limited $G\beta\gamma$ binding affinity, failed to accelerate current desensitization.
Measuring simultaneously membrane fluorescence signals from cells expressing GFP-GRK2 and GIRK activity under TIRF microscopy and electrophysiological recordings, respectively, show that GFP-GRK2 membrane recruitment and GIRK desensitization occurs simultaneously.
We suggest that GPCR activation induces GRK2 recruitment to the membrane, where it competes with GIRK for $G\beta\gamma$, to result in short-term current desensitization. The results will be discussed in terms of a novel mechanism of selective regulation of effectors by GPCRs.

2392-Pos Board B362

Rhythmic Control Of Atrial GIRK Channel Function By PKC

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G-protein-gated inwardly rectifying K^+ (GIRK) channels play a key role in the regulation of beat to beat variability of the heart (Wickman et al., 1998), yet the mechanisms that control this variability are not well understood. We hypothesized that different kinases known to regulate GIRK channel function might generate rhythmic changes in GIRK channel sensitivity to G protein stimulation in a ''tug of war'' manner with the PP1 and PP2A phosphatases associated with GIRK channels (Nikolov and Ivanova-Nikolova, 2004). To test this hypothesis we recorded the activity of single GIRK channels excised from the membrane of atrial myocytes in the presence of purified $G\beta\gamma$ and PKC. As previously reported, PKC abolished the activity of the canonical 35-pS GIRK1/4 channels. In contrast, PKC induced rhythmic activity of the small conductance GIRK (scGIRK) channels, residing in the atrial membrane. This rhythmic activity of scGIRK channels arises from constant concentration of $G\beta\gamma$, PKC and ATP and greatly expands the dynamic repertoire of the signaling system. PKC has pivotal role in signal transduction as modulator of the amplitude of protein function. Our data reveal that in addition to its canonical role in signaling pathways, PKC has the ability to assemble molecular clocks out of common membrane components expanding the dynamic nature of cellular signaling.

2393-Pos Board B363

Evidence for a Discrete Alcohol Pocket Mediating GIRK Channel Activation

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Alcohols can activate G protein-gated inwardly rectifying K (GIRK) channels but the molecular mechanism is not well understood. To investigate the possibility of a physical alcohol pocket located in the cytoplasmic domain of GIRK channels, we used a crystal structure of related IRK1 channel containing a bound alcohol (2-methyl, 2-4-pentanediol - MPD) and structure-based mutagenesis of GIRK2 and GIRK4 channels combined with patch-clamp electrophysiology. In transiently transfected HEK293 cells, both wild-type GIRK2 and GIRK4 channels were activated by 100 mM ethanol, MPD and 1-Propanol. Replacing a conserved Leucine (GIRK2-L257 / GIRK4-L252) in the betaD-betaE region of the cytoplasmic domain with bulkier Tyrosine or Tryptophan led to significant attenuation or loss of alcohol-dependent activation for both GIRK2 and GIRK4 channels. Constitutively open channels, such as IRK1 and GIRK2-PIP_2 (engineered to bind PIP2 with high affinity), on the other hand, were inhibited by ethanol, 1-propanol, 1-butanol and MPD. Mutating the homologous Leucine in IRK1 (L257) or in GIRK2-PIP2 channels did not alter the sensitivity to inhibition by these alcohols, suggesting a second site in the channel is involved in inhibition by alcohols. Consistent with this, mutagenesis of the extracellular pore-helix of GIRK4 and GIRK2-PIP_2 channels reduced the sensitivity to alcohol-mediated inhibition. Interestingly, mutation of the conserved Leucine (L257/L252) in the betaD-betaE domain also disrupted G protein-dependent activation, suggesting a common mechanism of activation by alcohols and G-proteins. Using our data and an analysis of high-resolution structures of inwardly rectifying K channels, we propose a novel model for alcohol activation of GIRK channels that is mediated by the cytoplasmic hydrophobic alcohol pocket.