step to +50 mV) by measuring inward rectifier I_{K1} tail currents (I_{tail}) at −75 mV. At room temperatures of ~21-23 °C the amplitude of I_{tail} current measured 9-10 minutes after the establishment of whole-cell configuration was essentially unchanged (93.6% of initial value), but declined to 53.5% upon application (within 2-5 minutes after the beginning of cell dialysis) of 1 mM cyanide, a blocker of oxidative phosphorylation (n=9 and 6, respectively; $p<0.01$). At 35-37 °C the I_{tail} was essentially preserved in intact cells, but its amplitude declined to ~30% within 4-5 minutes after the establishment of whole-cell configuration. In all cases no significant changes in either outward potassium currents or I_{K1} were observed, strongly suggesting that changes in t-tubular volume/structure are responsible for the observed effects. Detubulation of myocytes using formamide-induced osmotic stress nearly completely removed I_{tail} currents as well as decline of inward I_{K1} caused by I_{K1}-dependent depletion of t-tubular potassium. Overall, the data provide strong evidence that changes in t-tubular volume/structure may occur on a short time scale and suggest mitochondrial dysfunction as one of the underlying causes.

3629-Pos

A Structural Model of a Kir Channel in the Open State Derived from Mutagenic Scanning of the Pore Gating Energetics

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Specific stimuli (e.g. intracellular pH, PIP2) cause Kir channels to undergo a reversible transition between the closed and open state. The precise rearrangement of the pore structure (e.g. the TM regions) during these gating transitions is currently unknown in Kir channels owning to the lack of an open state crystal structure and validating functional data. We employed systematic alanine scanning mutagenesis on the entire Kir1.1 pore structure (residues 51 - 192) and determined the IC50 values for pH inhibition for each mutant. We reasoned that a shift in the IC50 value should represent a change in the free energy of the open state relative to the closed state assuming that the mutations did not affect the cytoplasmatic pH sensor directly. We identified 26 mutations that produced a marked shift in the pH sensitivity, intriguingly, 24 of these mutations increased the pH sensitivity suggesting that the open state is structurally more optimised than the closed state. Specifically, we expected that in the open state the IC50 shifting residues would interact with each other explaining why the alanine substitution would preferentially destabilise this state. We used this expectation as conceptual basis to develop scoring methods to evaluate structural models of Kir1.1 in the open state that we generated from existing crystallographic open state structures of other K+ channels (KvAP, NaK, KirBac3.1) using homology modelling and MD simulations. This analysis revealed an excellent agreement of our functional data with one particular open state model of Kir1.1. In this model more than 80% of the IC50 shifting residues are part of a tightly packed network of interacting residues that largely disintegrates upon channel closure.

3630-Pos

pH-Dependent Gating Mechanism of Kir2.1 Inward Rectifier K^+ Channel Independent of Polyamine and Magnesium Block

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Kir2.1 inward rectifier K^+ channel shows a strong inward rectification due to a voltage-dependent block of the channel pore by intracellular cations, such as polyamines and Mg^{2+}. In this study, we conducted experiments using inside-out patch membranes and found that Kir2.1 channel exhibits an extremely slow, voltage-dependent gating that depends on the cytoplasmic pH in the acidic range. This gating seemed to be unrelated to the block induced by polyamines that remained trapped near the cytoplasmic pore or by Mg^{2+} or impurities of EDTA contaminated in the cytoplasmic solution. Acidification of the cytoplasmic solution did not markedly affect the polyamine block of the wild-type Kir2.1 channel, indicating that the acidic residues lining the Kir2.1 pore (e.g. D172, E224 and E299), whose negative charges are known to contribute to polyamine binding sites, were not neutralized at acidic pHs. Thus, these negative charges did not seem to confer the pH sensitivity of the gating. However, when Kir2.1 channels bearing a mutation at these residues were tested, neutralization of D172 in the transmembrane region abolished the pH-dependent gating. The findings suggest that the gating may be caused by a pore block by an unknown molecule, bearing a positive charge at acidic pHs.

3631-Pos

The Site for Docking Cations in Cytoplasmic Domain of Inward Rectifier K^+ Channels

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Inward rectifier K^+ (Kir) channels participate in various cellular phenomena by regulating the membrane potentials. The Kir channel is a tetrameric assembly, consisting of two discrete domains, transmembrane and cytoplasmic domains. The channel possesses a long pore at the center of the assembly for the ion conduction. A number of studies have been reported to analyze how amino acids along the pore contribute to the ion conduction and inward rectification. However, it is still unclear how permeable cations interact with the residues at the cytoplasmic domain. In the crystal structure of cyoplasmic domain of Kir3.2, we observed a strong electron density at the cytoplasmic pore. When the crystals were soaked into Ba^{2+}-containing solution, an anomalous signal derived from Ba^{2+} was detected at the position corresponding to the electron density. This indicates that the strong electron density is accounted for by Mg^{2+} in the crystallization solution. The Mg^{2+} was surrounded by Glu236 and Met313 from four subunits. The pair of the residues is conserved among strong inward rectifiers, suggesting that the pair creates the site specific for the strong inward rectification. Branched and neutral amino acids substituted for Met313 conferred the weak rectification on the mutants and the mutation at Glu236 modulated the rectification property. Thus, the properties of the side chains at positions 236 and 313 are crucial for the conduction. On the other hand, the introduction of the pair of residues to weak inward rectifier Kir4.1 did not affect on the rectification property. The cytoplasmic pore is reported to provide an electrostatic environment for the accumulation of cations. The Mg^{2+}-docking site at the cytoplasmic pore of Kir3.2, therefore, seems to create the local environment for the stabilization of cations specific for the strong inward rectifiers.

3632-Pos

Voltage Dependence of Kir2.1 Block by Intracellular Spermine

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Strong voltage sensitivity of inward-rectifier K+ (Kir) channels, such as Kir2.1, has been hypothesized to arise primarily from an intracellular spermine molecule displacing K+ ions from the wide, intracellular part of the ion conduction pore outwardly across the narrow ion selectivity filter. This hypothesis anticipates: i) that mutations intracellular to the ion selectivity filter can abolish any high-affinity spermine block, and ii) that the blocker can force essentially unidirectional K+ movement in a pore region generally wider than the combined dimensions of the blocker plus a K+ ion. We find that simultaneous mutation of five residues, all located internal to the selectivity filter, abolishes specific spermine block. Thus, the selectivity filter itself evidently must have little inherent affinity for spermine. We also find that a constriction near the intracellular end of the pore, acting as a gasket, prevents K+ ions from bypassing the blocker. This heretofore unrecognized gasket ensures that the blocker can effectively displace K+ ions across the selectivity filter to generate exceedingly strong voltage sensitivity.

3633-Pos

A Multicistronic 2a-Peptide-Based Vector Encoding for Ci-VSP and a Pair of FRET Sensors to Study Effects of PIP_2-Depletion on Receptor-Activated GIRK Current

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Ci-VSP, a voltage-activated phosphatase, which dephosphorylates PI-(4,5)-bisphosphate (PIP_2) to PI(4)P combined with fluorescent (FRET) probes for optical monitoring of changes in plasma membrane PIP_2 is an emerging tool to study regulation of ion channels and transporters by PIP_2 (Murata et al., Nature 43, 2004). In the present study we used HEK239 cells to express GIRK1/GIRK4 channels by means of a conventional bi-cistronic vector containing an IRES sequence. The channel could be activated by a co-expressed A_1 receptor. For expression of Ci-VSP and a pair of FRET-generating PIP_2 binding probes (PH-PLCδ1-CFP and PH-PLCδ1-YFP) a multicistronic vector was constructed. This vector contained the cDNAs for the phosphatase and the fluorescent PH-domains separated by viral 2A-peptide sequences in a single ORF. The 2A-sequences result in cotranslational dissociation of the polyprotein while allowing translation to continue (de Felipe et al. JBC 278, 2003). Depolarizations to + 60 mV of variable duration (1 to 10 s) resulted in reductions in FRET ratio, indicating depletion of PIP_2. Concomitantly, adenosine-activated GIRK current was reduced. The onset of current inhibition was faster than the onset of

reduction in FRET, whereas upon repolarization the latter recovered faster ($t_{1/2}$ ~ 10 s) as compared to recovery of the current. This suggests the PIP_2 affinity of the regulatory binding sites on the channel subunit(s) to be lower than the affinity of the PLCδ1 PH-domain. Our data demonstrate versatility of 2A-peptide based expression vectors for manipulation and quantifying membrane phosphoinositides in cell lines and primary cells.

3634-Pos

Caveolin-1 Inhibits Vascular K_{ATP} Channels by Modulating Channel Sensitivity to MgADP

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Caveolae are small vesicular invaginations of the plasma membrane enriched in cholesterol, sphingolipids and the cholesterol-binding protein, caveolin [1]. Aside from roles in caveolae formation and stability, caveolins interact with many caveolae-localised molecules via an N-terminal region known as the caveolin scaffolding domain. One of the many signalling proteins that reside in caveolae and interact with caveolins is the vascular ATP-sensitive potassium (K_{ATP}) channel [2], an ion channel important in the control of smooth muscle contractility and so blood flow. Here we investigate the potential role of caveolin in regulating K_{ATP} channel behaviour.

Pinacidil-evoked recombinant whole-cell K_{ATP} (Kir6.1/SUR2B) currents recorded in HEK293 cells stably expressing caveolin-1 (69.6 ± 8.3pA/pF, n=8) were found to be significantly smaller than currents recorded in caveolin-null cells (179.7 ± 35.9pA/pF, n=6; $p<0.05$), indicating that interaction with caveolin-1 may inhibit channel activity. The addition of a peptide corresponding to the caveolin-1 scaffolding domain to the pipette-filling solution had a similar inhibitory effect on whole-cell recombinant K_{ATP} currents. In cell-attached patch clamp recordings, the presence of caveolin-1 significantly reduced channel open probability and the amount of time spent in a relatively long-lived open state. Sensitivity of the channel to its physiological regulator MgADP was significantly altered by caveolin-1 and can explain these changes in channel kinetic behaviour.

Our findings suggest that interaction with caveolin-1 has an inhibitory effect on arterial-type K_{ATP} channel activity that may be important in both the physiological and pathophysiological control of vascular function.

1. Parton & Simons (2007). *Nat Rev Mol Cell Biol* **8,** 185-194.
2. Sampson *et al*. (2004). *Circ Res* **95,** 1012-1018.

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3635-Pos

Differential PKC Isoform Regulation of K_{ATP} Channel Trafficking and Function

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Pancreatic ATP sensitive potassium (K_{ATP}) channels play an important role in insulin secretion, linking the metabolic state and excitability of the beta cell. K_{ATP} channel function is under complex regulation by protein kinase C (PKC) with both activation and inhibition reported. To address this apparent contradiction we have employed a range of cell biological, electrophysiological and biochemical techniques to elucidate the mechanisms of PKC regulation of pancreatic K_{ATP} channels. Acute PKC activation (5 min) led to a large increase in K_{ATP} currents recorded from HEK cells stably expressing Kir6.2 and SUR1. This increase in currents was accompanied by increased colocalisation of K_{ATP} channels with classical PKC isoforms and was sensitive to inhibition by the classical PKC inhibitor Gö6976. Prolonged (1 hour) activation of PKC however led to a significant reduction in K_{ATP} channel currents accompanied by a loss of channels from the cell surface and an increase in their lysosomal degradation. Decreased K_{ATP} channel surface expression was not sensitive to Gö6976 indicating novel PKC isoforms. Both dominant negative PKC epsilon and PKC epsilon inhibiting peptides were able to inhibit the PKC mediated decrease in channel surface density. These data suggest that classical and novel PKC isoforms differentially regulate the function and trafficking of pancreatic K_{ATP} channels comprising Kir6.2 and SUR1. Further work is underway to assess the physiological significance of differential PKC isoform activation following pancreatic beta cell stimulation.

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3636-Pos

Intracellular Mechanisms Responsible for PKG Stimulation of ATP-Sensitive Potassium Channels

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The activity of the ATP-sensitive potassium (K_{ATP}) channel, a high-fidelity metabolic sensor that couples cellular metabolic status to membrane excitability, is modulated by a variety of post-translational mechanisms. Findings obtained from our recent studies suggest that activation of cGMP-dependent protein kinase (PKG) enhances the function of neuronal K_{ATP} channels via intracellular signaling and that generation of reactive oxygen species (ROS) is required for PKG stimulation of the channel. In the present study we further investigated the potential role of intracellular calcium and camodulin in mediating the modulatory effect of PKG and ROS. By performing single-channel recordings in transfected human embryonic kidney (HEK) 293 cells, we found that the increase in the single-channel activity of Kir6.2/SUR1 channels (a neuronal/pancreatic K_{ATP} channel isoform) by activation of PKG in cell-attached patches was abrogated by BAPTA-AM, a membrane-permeable calcium chelator and SKF-7171A, a membrane-permeable calmodulin antagonist. Activation of PKG was achieved by bath application of zaprinast, a cGMP-dependent phosphodiesterase inhibitor, and the specificity of PKG activation was verified by selective blockade by PKG- but not PKA-specific inhibitors. Moreover, bath application of H_2O_2 dose-dependently increased the activity of Kir6.2/SUR1 channels in cell-attached but not inside-out patches, and the stimulatory effect was attenuated by suppression of calmosulin. Altogether, our findings suggest that PKG stimulates neuronal K_{ATP} channels via ROS generation and subsequent activation of calmodulin. The cGMP/PKG/ROS/calmodulin signaling cascade may play an important role in controlling neuronal excitability, neurotransmitter release and neuroprotection against ischemic injury, by modulating the function of plasma-membrane K_{ATP} channels.

3637-Pos

Investigating the PIP2 Binding Site in Kir Channels Via Multi-Scale Biomolecular Simulations

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Mammalian inwardly rectifying potassium (Kir) channels are activated by the anionic lipid, Phosphatidylinositol bisphosphate (PIP2). In this study, coarse-grained simulations followed by atomistic molecular dynamics have revealed the interactions made by PIP2 molecules with Kir channels. The three channels investigated are X-ray structures of KirBac1.1 and the Kir3.1-KirBac1.3 chimera, and a homology model of Kir6.2. Coarse-grained simulations of the Kir channels in PIP2-containing POPC lipid bilayers identified the PIP2 binding site on each channel. These models of the PIP2/channel complexes were refined by conversion to an atomistic representation followed by molecular dynamics simulation in a lipid bilayer. All three channels were revealed to contain a conserved binding site at the N-terminal end of the slide (M0) helix, at the interface between adjacent subunits of the channel. This binding site agrees with known functional data and is in close proximity to the site occupied by a detergent molecule in the Kir chimera channel crystal. Polar contacts in the coarse-grained simulations agree well with H-bonding interactions between the channels and PIP2 in the atomistic simulations, enabling identification of key sidechains, which are primarily basic in nature. Notable differences within the KirBac1.1 and Kir6.2 binding sites are apparent; providing hypotheses for why PIP2 activates Kir6.2 channels whilst inhibiting the opening of KirBac1.1 channels.

3638-Pos

Functional Characterization of the Andersen-Tawil Syndrome Associated Mutation (M307I) in *KCNJ2*

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Background: Inward rectifier potassium channels play a key role in setting and maintaining the resting membrane potential and regulating excitability in various tissues. Kir2.x subfamily members mediate the cardiac inward rectifier potassium current (IK1). KCNJ2 encodes Kir2.1, pore-forming alpha subunit of cardiac IK1, and the mutations in this gene are associated with type 1 Andersen-Tawil Syndrome (ATS1). The aim of this study was to characterize the electrophysiological features of a Kir2.1 missense mutation, M307I, found previously in a Korean family with ATS. Methods and Results: Site-direct mutagenesis and heterologous expression system was used for functional study. Kir2.1-M307I and Kir2.1 or Kir2.2 wild type (WT) channels were expressed individually and together in HEK293 cells to measure IK1 by voltage clamp. After 24h of incubation, the whole cell patch clamp technique revealed a 100% loss of outward current over the voltage range of −60 mV to −20 mV for Kir2.1-M307I alone and when co-expressed with WT channels (current density at −40 mV was 9.6 ±3.6 pA/pF for Kir2.1-WT and 3.0 ±1.6 pA/pF for Kir2.2-WT, n=4-10, $p<0.001$). The inward current over the voltage range of −140 mV to −80 mV was also reduced significantly in mutant co-expressed with WT channels. We further tested an action potential (AP) voltage clamp