Many diabetogenic mutations (*Endocrine Rev* 2009;29:265) map to the canonical, N-terminal, submembrane (*BBRC* 1999;255:231) ''slide'' M0 helix of KCNJ11 ($K_{IR}6.2$). To clarify the principal biophysical mechanism of their action, inhibiting insulin release, I analyzed effects of the first reported severe Neonatal Diabetes (ND with epilepsy and developmental delay) mutation and 18 other mutations, V59X, in M0 on macroscopic and unitary currents through ABCC8(SUR1)-containing ATP-sensitive potassium (K_{ATP}) channels, reconstituted in mammalian cells lacking endogenous SUR or K_{IR}. Several V59X decreased, and no V59X increased or abolished, functional expression (N) of the neuroendocrine-type adenine nucleotide sensors. This a) indicated that the effect of any possible ND V59X-induced decrease in N on V_m in humans is overruled by the mean open channel probability(P_O)-increasing effect, explaining insufficient insulin release due to hyperpolarization of insulin producing cells, and b) allowed complete analysis of relationships between the physical properties of the side chain in the middle of M0 and P_O, its sensitivity to nucleotides, and single-channel gating kinetics. The established relationships are consistent with the results of molecular modeling and molecular dynamics simulation of severe ND K_{ATP} pores and strongly suggest that a ligand-independent stabilization of the active (burst) state with conformations without specific, micromolar affinity for inhibitory ATP, is the principal mechanism of pathogenic hyperactivity of K_{ATP} with mutations in M0, the small domain proposed to play a big role in gating of $K_{IR}6$ and their relatives.

I thank the National Institutes of Health for funding and G. Zhao for technical assistance.

3644-Pos

Identification of the Alcohol Activation Site in GIRK Channels

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In addition to G proteins, ethanol can activate G protein-gated inwardly rectifying K (GIRK) channels. The mechanism underlying GIRK channel activation by alcohol is not well understood. Based on a crystal structure of a related IRK1 channel which contains the alcohol (2-methyl,2-4-pentanediol- MPD) bound to a cytoplasmic hydrophobic pocket, we used structure-based mutagenesis and patch-clamp electrophysiology to investigate the role of the homologous alcohol pocket in GIRK2 channels. In HEK293T cells transfected with GIRK2 cDNA, both ethanol and MPD activated GIRK2 channels. Replacing a conserved Leucine (L257) in this pocket with a bulkier Tyrosine or Tryptophan led to significant attenuation or loss of alcohol-dependent activation of GIRK2 channels, suggesting these larger hydrophobic side-chains filled the pocket. Based on structure and functional evidence, we conclude that this hydrophobic pocket is the site for alcohol activation of GIRK channels. We hypothesized that tethering a hydrophobic group near the pocket might mimic alcohol mediated activation of the channel. To test this idea, we introduced a S246C mutation in a Cysteine-less GIRK2 channel and examined the effect of bath applied MTS-Benzene. Application of 10 micromolar MTS-Benzene dramatically increased the size of basal GIRK currents by 336+66% n=5. This rapid activation was reversed by application of reducing agent DTT (10 mM), indicating a disulfide bond had formed. In addition to the change in basal current, MTS modification of S246C channel altered the rank order for alcohol activation -with significantly less activation by the larger alcohol MPD. These results suggest that attachment of a bulky hydrophobic amino acid near the hydrophobic alcohol-binding pocket can produce sustained activation of the channel by associating with the activation site. These experiments provide a launching point to study molecular events at this hydrophobic pocket that lead to activation of GIRK channels.

3645-Pos

The Outer Transmembrane Domain is Involved in a Slow Voltage-Dependent Gate in a K+ Channel

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Many voltage-dependent channels activate in a time-dependent manner. A lesson on the mechanism of this slow gating can be learned from the small viral K+ channel Kcv. This channel, with a monomer size of 94 amino acids, has the advantage of being truly minimal; it consists of an outer (TM1) and an inner (TM2) transmembrane domain and a pore loop with minimal N and C termini. Kcv reveals in Xenopus oocytes a time-dependent inward rectification. This slow activating component is absent when the channel is expressed in HEK293 cells. It can can be regained in the latter expression system when Pro13, the amino acid, which marks entry of TM1 into the membrane, is replaced by an alanine. Single channel recordings of Kcv-P13A reveal that the open probability is much higher than in the wild-type.

A similar gain in function is obtained when TM1 is extended by insertion of alanine downstream of Pro13. The region in which an extension of TM1 promotes this gain of function shows high flexibility in molecular dynamics (MD) simulations of Kcv. The idea that flexibility is related to slow gating is supported by the temperature sensitivity of the kinetics. In mutants with an extended TM1 the time constant of activation is strongly temperature-dependent, decreasing at high temperature.

Experimental and theoretical data supports a model in which the movement of the N-terminal part of TM1 is involved in time dependent gating. MD simulation shows transient salt bridge patterns between TM1 and TM2 controlling the entry of ions into the cavity. We speculate that formation and disruption of these salt bridges is part of the slow gating process and that an increased flexibility of TM1 modulates the frequency for salt bridge formation.

3646-Pos

The Lipid Dependence of Purified and Reconstituted Kir2.1 and Kir2.2

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Ion channels are embedded in the membrane bilayer and are known to be regulated by their lipid environment. Insights on the structural basis of channel-lipid interactions have been gained by recent potassium channel crystal structures that reveal bound lipid or detergent molecules. However, efforts to define the lipid dependence of channel activity have been limited to cellular expression systems, in which the membrane composition cannot be fully controlled. We have expressed and purified functional human Kir2.1 and Kir2.2 from S. cerevisiae, and characterized the phospholipid dependence of channel activity in a liposomal 86Rb+ flux assay. Reconstituted Kir2.1 and Kir2.2 require incorporated PIP2 for activity and are maximally active in 0.1-1% PIP2 on a background of 3:1 POPE:-POPG. This provides definitive evidence that eukaryotic Kir channels are directly activated by PIP2 without any intermediary components. Interestingly, Kir2.1 and Kir2.2 are minimally active in ~1% PIP2 on a POPE (neutral) background, and are activated by increasing amounts of POPG (1 negative charge) or other anionic phospholipids. By contrast, the prokaryotic inward rectifier, KirBac1.1, shows no phospholipid dependence of activity, except potent inhibition by PIP2 (1), DGS-NTA, cardiolipin and oleoyl CoA. Our data suggest that the site of action for this secondary regulation by anionic phospholipids in Kir2.1 and Kir2.2 is distinct from the cytoplasmic PIP2 binding site. This study represents the first description of the lipid dependence of activity for recombinantly-expressed, purified eukaryotic ion channels in liposomes, and demonstrates that Kir2.1 and Kir2.2 have two lipid requirements for activity: a high affinity requirement that is specific for PIP2, and a low affinity requirement that is relatively non-specific for anionic phospholipids.

1. D. Enkvetchakul, I. Jeliazkova, C. G. Nichols, J.Biol.Chem. 280, 35785 (2005).

3647-Pos

Direct Regulation of Prokaryotic Kir Channel by Cholesterol

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Our earlier studies have shown that channel activity of Kir2 sub-family of inward rectifiers is strongly suppressed by the elevation of cellular cholesterol. The goal of this study is to determine whether cholesterol suppresses Kir channels directly. To achieve this goal, purified prokaryotic Kir (KirBac1.1) channels were incorporated into liposomes of defined lipid composition and channel activity was assayed by $^{86}Rb^+$ uptake. Our results show that $^{86}Rb^+$ flux through KirBac1.1 is strongly inhibited by cholesterol. Incorporation of 5% (mass Chol/PL) cholesterol into the liposome suppresses $^{86}Rb^+$ flux by >50% , and activity is completely inhibited at 12-15%. However, epicholesterol, a stereoisomer of cholesterol with similar physical properties, has significantly less effect on KirBac-mediated $^{86}Rb^+$ uptake than cholesterol. Furthermore, analysis of multiple sterols suggests that cholesterol-induced inhibition of KirBac1.1 channels is mediated by specific interactions rather than by changes in the physical properties of the lipid bilayer. In contrast to the inhibition of KirBac1.1 activity, cholesterol had no effect on the activity of reconstituted KscA channels (at up to 250 μg/mg PL). Taken together, these observations demonstrate that cholesterol suppresses Kir channels in a pure protein-lipid environment and suggest that the interaction is direct, and specific.

3648-Pos

Functional Reconstitution of a GIRK1-Chimera and its Regulation by the βγ Subunits of G Proteins

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The molecular details of ion channel regulation by G proteins remain unknown. A first step in this direction is to define the characteristics of interacting proteins of known structure in isolation and in complex form. The three-dimensional structure of a GIRK1-chimera determined by single particle electron microscopy at 25Å is consistent with the crystal structure (Nishida et al., 2007, EMBO J 26:4005-15). We have functionally reconstituted this GIRK1-chimera into a 1:1 ratio of phosphatidylethanolamine to phosphatidylserine planar lipid bilayers. The GIRK1-chimera produces a conductance of approximately 23 pS that shows Mg^{2+}-dependent inwardly rectifying K^+ currents and an absolute requirement on the presence of phosphatidylinositol-4,5-bisphosphate for activation. These currents are blocked by PIP_2 antibody and poly-lysine applied from the cis but not the trans side. Moreover, the channel shows a high affinity for diC8-PIP_2 (EC_{50} ~ 7.5 μM). GIRK1-chimera channel currents are blocked by Ba^{2+} and the GIRK peptide blocker tertiapin when applied from the trans but not the cis side. Interestingly, Gβγ applied from the cis side inhibits GIRK1-chimera currents and shifts phosphoinositide sensitivity by decreasing the apparent affinity to PIP_2. This is in contrast to the Gβγ effects on full-length GIRK1* channels assayed in *Xenopus* oocytes or planar lipid bilayers.

3649-Pos

Expression and Purification of Recombinant Human Inward Rectifier K^+ (KCNJ) Channel

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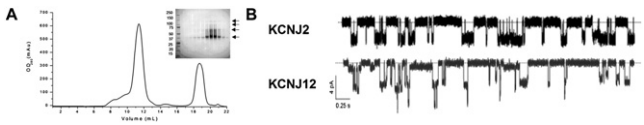
Inward rectifier potassium (KCNJ) channels regulate vital cellular processes including cell volume, electrical excitability, and insulin secretion. Dysfunction of different isoforms has been linked to numerous diseases including Bartter's, Andersen-Tawil, Smith-Magenis Syndromes, diabetes, and epilepsy. We succeeded in expressing 10 of 11 human KCNJ channels tested in *Saccharomyces cerevisiae* under Gal1-inducible promotion. GFP-fusion proteins are located in the plasma-membrane, suggesting the protein is correctly folded and trafficked. Following large scale expression of Kir2.x family members, a 2-step purification process can be used to isolate protein to >95% in a mono-dispersed form (Fig.1A). $^{86}Rb^+$ flux assays and patch clamp analysis on reconstituted proteins confirm the functionality of the purified proteins as inward rectifier potassium channels. For KCNJ2 (Kir2.1) and KCNJ12 (Kir2.2) channels, the unitary conductance in 150mM symmetrical [K^+] (~33pS and ~40.5pS, respectively, Fig.1B at −100mV), sensitivity to spermine block, and activation by $PIP(4,5)_2$ resemble those observed in eukaryotic membranes. The high-level purification and reconstitution of these proteins makes feasible not only ongoing biochemical and structural analysis of eukaryotic KCNJ channels, but also the analysis of channel function in the absence of modulator proteins, and in membranes of defined composition.



3650-Pos

Purified Hetero-Tetramers of the Potassium Channel Kcv Revealing Independent Subunit Contribution to the Tea Block

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Tetraethylammonium (TEA) is a common molecular probe in detecting potassium channel blocking. The external TEA binding affinity has been proposed to be highly related to the aromatic residue located at the outer mouth of the potassium channels such as Y82 in KcsA and Y449 in Shaker, probably due to the π-cation interaction between TEA and the aromatic side chain. In this report, we identified the highly sensitive TEA block for the chlorella virus-encoded Kcv, a miniature model K+ channel that only consists of 94 amino acids with two transmembrane domains and a conservative selectivity filter. By mutagenesis screening at Leu70 of Kcv, which is equivalent to the TEA site Y82 in KcsA, we found substitution of Leu70 to all other amino acids including Tyr, Phe and His will reduce the TEA affinity, suggesting a more complicated mechanism beyond cation-π interaction involved in TEA blocking. We further developed a novel functional stoichiometric approach to exploring how each individual subunit contributes to the TEA binding. We co-expressed the mutant Kcv and a mass-tagged wild-type Kcv, to form hetero-tetramers that can be electrophoretically separated. Because Kcv is able to retain the channel-forming function in detergent SDS [*FEBS Lett.* 581, 1027-1034 (2007)], we can purify all types of hetero-channels directly from the SDS gel, and subject to single channel recording. Through this approach, we established a linear correlation between the free energy for TEA blocking and the number of mutant subunits in a tetramer, which infers that each subunit independently interacts with one ethyl group of TEA and contributes equal energy to the overall TEA affinity. The functional stoichiometric approach we developed with purified hetero-channels can be applied to the mechanism study of many K+ channel drugs and inhibitors.

Ligand-gated Channels

3651-Pos

Desensitization Contributes to the Postsynaptic Response of Ionotropic Receptors; A Comparative Study of Cys-Loop, Purinergic, and Glutamate Receptor-Channels

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All naturally-occurring ionotropic receptors desensitize significantly in the continuous presence of a sufficiently high concentration of agonist. However, the neurotransmitter lifetime in the synaptic cleft is limited by diffusion, neurotransmitter reuptake, and/or enzymatic cleavage, to an extent such that desensitization does not appreciably occur during the agonist pulse. Nevertheless, channels remain prone to desensitization during the much longer interpulse intervals while they deactivate. As a result, it is of interest to determine the extent to which ligand-gated ion channels (LGICs) known to participate in fast synaptic transmission undergo desensitization upon ligand removal, since entry into these refractory states would progressively decrease the postsynaptic-current response. To address this problem, we have applied high-frequency trains of brief (approximately 1 ms) agonist pulses to outside-out membrane patches expressing these LGICs; receptors under study include the rat purinergic P2X receptor, the rat AMPA-type glutamate receptor, the rat gamma-aminobutyric acid receptor (GABAR), the human glycine receptor (GlyR), and the human ganglionic (alpha3-beta4) and mouse-muscle nicotinic acetylcholine receptors (nAChRs). Our results indicate that all tested receptors exhibit increasingly reduced peak responses in a train-frequency- and receptor-dependent manner, consistent with the notion that the extent of desensitization upon deactivation is substantial. These findings suggest that a) receptor desensitization may contribute to limit the in-vivo postsynaptic response mediated not only by glutamate receptors (which has been proposed earlier), but also by all of the other ionotropic receptors studied here, and b) that the occurrence of desensitization cannot be neglected (as it often is) in attempts to characterize the kinetic behavior of these channels.

3652-Pos

Effects of Protons on Macroscopic and Single-Channel Currents Mediated by the Human P2X7 Receptor

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Human P2X7 receptors (hP2X7Rs) belong to the P2X family, which opens an intrinsic cation channel when challenged by extracellular ATP. hP2X7Rs are expressed in cells of the inflammatory and immune system. During inflammation, ATP and protons are secreted into the interstitial fluid. Therefore, we investigated the effect of protons on the activation of hP2X7Rs. hP2X7Rs were expressed in *Xenopus laevis* oocytes and activated by the agonists ATP or benzoyl-benzoyl-ATP (BzATP) at different *pH* values. The protons reduced the hP2X7R-dependent cation current amplitude and slowed the current deactivation depending on the type and concentration of the agonist used. These effects can be explained by (i) the protonation of ATP, which reduces the effective concentration of the genuine agonist, free ATP^{4-}, at the high- and low-affinity ATP activation site of the hP2XR, and (ii) direct allosteric inhibition of the hP2X7R channel opening that follows ATP binding to the low-affinity activation site. Due to the hampered activation via the low-affinity activation site, a low *pH* (as observed in inflamed tissues) leads to a relative increase in the contribution of the high-affinity activation site for hP2X7R channel opening.

3653-Pos

P2X7 Receptor-Mediated Disruption of the Plasma Membrane and Endoplasmic Reticulum Morphology and Cell Survival

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The cation-conducting P2X7 receptor channel (P2X7R) operates as a cytolytic and apoptotic nucleotide receptor but also controls sustained cellular responses, including cell growth and proliferation. However, it has not been clarified how