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Diverse Regulation of the Neuronal G-Protein Gated K^+ Channel (GIRK), GIRK1 and GIRK2 by Gα and Gβγ

Moran Rubinstein[1], Shai Berlin[1], Tal Keren-Raifman[1], Sagit Peleg[1], Carmen Dessauer[2], Tatiana Ivanina[1], Nathan Dascal[1].
[1]Tel Aviv University, Tel Aviv, Israel, [2]University of Texas-Houston, Houston, TX, USA.

G-protein activated K^+ channels (GIRK, Kir3) mediate postsynaptic inhibitory effects of neurotransmitters via activation of G-protein coupled receptors, followed by activation of $G_{i/o}$ proteins and direct binding of Gβγ. The main neuronal GIRK channel is composed of GIRK1 and GIRK2 heteromers. These channels express in various regions of the brain. Unlike GIRK1, GIRK2 can also form function homomeric channels predominantly expressed in the substantia nigra. *In vitro* protein interaction studies showed that the binding of the whole cytosolic domain of GIRK1 to $G\alpha_{i3}^{GDP}$ or $G\alpha_{i3}^{GTP}$ was enhanced by the presence of Gβγ. This increment was not observed with GIRK2, implying subunit specific modulated by G-proteins.

Functional implication of these diversions were explored using homomeric GIRK1* (a pore mutant that forms functional homomers) and GIRK2 channels in *Xenopus* oocytes. In addition, two $G\alpha_{i3}$ mutants were utilized to simulating the GTP/GDP bound states: ''constitutively active'' $G\alpha_{i3}$Q204L (''QL''; poor GTPase) and ''constitutively inactive'' $G\alpha_{i3}$G203A (''GA'') which forms a stable complex with Gβγ. GIRK2 behaved like a ''classical'' Gβγ effector, demonstrating very low basal activity and strong Gβγ-dependent activation, while Gα expression was without effect. GIRK1* exhibited large basal currents and no response to coexpressed Gβγ, whilst retaining activation by agonist. Furthermore, in excised patches GIRK1* homomers displayed a reverse correlation between the basal activity and the Gβγ evoked currents. $G\alpha_{i3}$GA restored the ability of Gβγ to activate GIRK1*, whereas $G\alpha_{i3}$QL elicited no effect.

These results suggest a specific role for GIRK1 as the scaffold for $G\alpha_i\beta\gamma$ within GIRK-G-protein signaling complex, while GIRK2 is the Gβγ sensitive, responsive subunit. Moreover, we suggest that GIRK1/2 may contribute to regulation of resting potential and excitability in neurons, whereas GIRK2 homomers serves as a high-gain neurotransmitter-induced inhibitory relay.

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Both "Constitutively-active" and "Inactive" $G\alpha_{i3}$ Mutants Interact with GIRK1/2 Heterotetramer

Shai Berlin, Tal Keren-Raifman, Moran Rubinstein, Tatiana Ivanina, Nathan Dascal.
Tel Aviv University, Tel Aviv, Israel.

Biophysical and imaging studies of the G-protein-inward-rectifying-K^+-channel (GIRK), point towards the existence of multiprotein complexes of channel, G-proteins and occasionally G-Protein-coupled-receptors (GPCR). It is established that GIRK directly interacts with the Gβγ subunits, before and after receptor activation, whereas GIRK's interaction with the $G\alpha_i$ subunit remains unresolved. We previously reported that GIRK is closely regulated by the $G\alpha_i$ subunit. Biochemically, we find that the full cytoplasmic domain of the channel (G1NC) binds $G\alpha_i$. This interaction is enhanced in the presence of Gβγ, with either GDP or GTPγS. Our findings demonstrate that both N- and C- termini of the channel, when associated with Gβγ, form a favorable 3D binding domain for the active and the inactive $G\alpha_{i3}$ subunit.

We assessed the interaction between GIRK and G proteins with fluorescence resonance energy transfer (FRET), using a doubly-labeled channel (DL-GIRK1), showing that the DL-GIRK channel acts as a sensitive reporter for the presence of G-proteins. DL-GIRK1 coexpressed with Gβγ displayed an increase in FRET, implying the nearing of N- and C-termini. Both phosducin and $G\alpha_{i3}$GA (''constitutively-inactive'' mutant, $G\alpha_{i3}$G203A) caused a dramatic decrease in both currents and FRET, probably by deviating Gβγ from its activation site. Nevertheless, coexpression of $G\alpha_{i3}$GA and Gβγ restored the channel's open conformation as reported by FRET and currents, whereas phosducin did not. An observed increase in FRET, with coexpressed $G\alpha_{i3}$Q204L (''constitutively-active'' $G\alpha_{i3}$ mutant) and Gβγ, strongly supports our biochemical findings of constitutive interaction. We tested the effect of Gαβγ on a doubly-labeled G-protein-insensitive-inward-rectifier-K^+-channel, Kir2.1 (DL-IRK1). DL-IRK1 failed to demonstrate any changes in FRET with coexpressed G-proteins.

Our findings imply that GIRK acts as the nucleator of the GIRK-Gα-Gβγ complex. Both active and inactive $G\alpha_{i3}$ remain bound to the channel, ensuring fast and specific activation and termination of the signal.

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Structural alterations in the cytoplasmic region of G protein-gated inward rectifier potassium channel, Kir3.2

Atsushi Inanobe[1,2], Takanori Matsuura[3], Haruki Nakamura[3,2], Atsushi Nakagawa[1], Yoshihisa Kurachi[1,2].
[1]Osaka University, Graduate School of Medicine, Suita, Japan, [2]Osaka University, The Center for Advanced Medical Engineering and Informatics, Suita, Japan, [3]Osaka University, Institute of Protein Research, Suita, Japan.

G protein-gated inward rectifier potassium (K_G) channel underlies the deceleration of the heartbeat upon vagal nerve stimulation and the formation of slow inhibitory postsynaptic membrane potential in neurons. The K_G channels are tetramers and either heteromeric or homomeric assembly of Kir3.1-Kir3.4 subunits and their splicing variants. Like the other inward rectifiers, the K_G channel possesses two distinct domains, a transmembrane domain and a cytoplasmic domain. The cytoplasmic domain of K_G channel containing either Kir3.2 or Kir3.4 is thought to interact with channel activators such as G protein βγ subunit, intracellular Na^+ and PIP_2, and control the channel gating at the transmembrane domain. However, it is not clear how such activators interact and cause the structural alteration in the cytoplasmic region of K_G channel. In this study, we prepared protein crystals of the cytoplasmic region of K_G channel subunit Kir3.2 in the presence or absence of its channel activator Na^+, and then compared both crystal structures. Essential conformational changes between two structures were observed around aspartate 228 on CD loop which was in the vicinity of the plasma membrane. The change in the structure affected on the interaction between N- and C-termini and yielded the different positions of β strand in N-terminus. The aspartate 228 is thought to be responsible for the Na^+-dependent activation, and the interaction between N- and C-termini is also known to be crucial for the regulation in the channel gating of inward rectifiers. These observations suggested that the K_G channel activator Na^+ caused structural alterations restricted at the membrane-facing area in the cytoplasmic region, leading to the regulation of the channel gating at the transmembrane domain.

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Analysis of GIRK Subunit Intracellular Domain Association and Channel Function

Erwin Gomez, Jennifer Hipp, **Radmila Sarac, Ph.D.**
Purdue University Calumet, Hammond, IN, USA.

The G protein coupled inwardly rectifying potassium (GIRK) channels are key mediators of cell excitability. In previous studies, we have identified important GIRK1 subunit residues that are critical to channel function and N- and C-terminal domain association. To further explore the specificity of these interactions and the functional implications, we have focused on determining if the nature of these associations is dependent upon a hydrophobic region in the N- and C-terminal domains of the GIRK1 and GIRK4 subunits. Mutations of the GIRK1 N- or C-terminal residue to the complementary residue did not significantly alter the carbachol-induced channel activation whereas mutation to a charged arginine greatly altered the association of domains and channel function. Similarly, mutation of the homologous GIRK4 subunit N- and C-terminal residues alters channel function and affects association between domains. Further analysis confirms that the C-terminal mutation to the complementary N-terminal residue preserved the termini association while mutation to a charged residue disrupts the association suggesting that this region is critical for interaction.

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Unique Role For The -5′ Position In The Carboxyl Terminus Of GIRK3 Channel In Determining Binding Specificity To The PDZ Domain Of Sorting Nexin 27

Bartosz Balana, Innokentiy Maslennikov, Steven M. Thomas, Joshua Tan, Witek Kwiatkowski, Senyon Choe, Paul A. Slesinger.
Salk Institute for Biological Studies, La Jolla, CA, USA.

We recently established that sorting nexin 27 (SNX27) regulates trafficking of neuronal GIRK2/3 but not the closely related IRK1 channels. This regulation is achieved by the interaction of the C-terminal PDZ binding motif (-SKV) of GIRK3 with the PDZ domain of SNX27. We also found that IRK1(-SEI) binds to PSD95-PDZ2 but not to SNX27-PDZ. Due to the similarity in the PDZ binding motif between IRK1 and GIRK3, we hypothesized that amino acids in the -4′ and -5′ position are important for determining PDZ binding specificity. Using in vitro binding assays, we discovered that exchanging the these amino acids, IRK1(-ESESKV) and GIRK3(-RRESKV), reversed the binding specificity, suggesting a critical role for -5′ glutamate for binding SNX27 and -5′ arginine for binding PSD95. Further mutagenesis in IRK1 revealed that aspartate or glutamine substitution at -5′ position, IRK1(-DRESKV) and IRK1(-QRESKV), promotes binding to SNX27-PDZ. Surprisingly, substitution with the large, hydrophobic tryptophan enhanced SNX27 binding more than the wild-type glutamate; IRK1(-WRESKV) was 5 ± 1.3 fold greater than IRK1(-ESESKV). We next investigated the consequence of altering the PDZ binding specificity using a clustering assay. CFP-tagged IRK1 or GIRK3 were co-expressed with YFP-tagged PSD95 or SNX27 in COS7 cells. Using TIRF microscopy to visualize

proteins at or near the cell membrane, we observed that mutant IRK1(-ESESKV) and wild-type GIRK3(-ESESKV) partially co-localized with SNX27 but did not form clusters. By contrast, mutant GIRK3(-RRESKV) and IRK1(-RRESKV) co-clustered with PSD95. We then used X-ray crystallography to solve the crystal structure of SNX27-PDZ complexed with ESESKV and are now using the structural data to correlate with binding data. These studies provide new details into the specificity of PDZ binding among class I PDZ binding motifs

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Hl-1 Cardiomyocytes As A Tool For The Study Of Regulation Of Kir3.1/Kir3.4 Channel Activity

Lia Baki[1], Aldo A. Rodriguez[1], Radda Rusinova[2], Charles D. Anderson[1], Diomedes E. Logothetis[1].
[1]Department of Physiology an Biophysics, Virginia Commonwealth University, Richmond, VA, USA, [2]Department of Physiology an Biophysics, Cornell University, New York, NY, USA.

The K_{Ach} channel slows the heart rate it in response to acetylcholine (ACh). Binding of ACh to the M2 Muscarinic receptor triggers Gβγ-mediated activation of the cardiac GIRK1/GIRK4 inwardly rectifying K channel, which is a heterotetrameric complex of the Kir3.1 and Kir3.4 subunits. Several reports, including work from our laboratory, suggest that phosphorylation events may be critical determinants of the above regulation. Apart from the heterologous expression of the channel subunits in various systems, primary atrial cultures have been so far the only available system for such studies. The development of a cardiomyocyte cell line (HL-1) which has the ability to continuously divide while maintaining a differentiated cardiac phenotype, prompted us to examine whether it could be used for studies on the regulation of GIRK1/GIRK4 channel activity. Here we report that HL-1 cells express both the Kir3.1 and Kir3.4 subunits, antibodies raised against each subunit co-immunoprecipitate the other subunit, the cells respond to ACh and growth factor receptor stimulation, show K_{Ach} currents and display electrophysiological properties characteristic of atrial K_{Ach}. Our data indicate that the HL-1 cell line provides a useful tool for the dissection of mechanisms regulating GIRK1/GIRK4 activity *in vivo*.

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Characterization Of Girk1 In Different Breast Cancer Cell Lines

Valerie Wagner, Astrid Gorischek, Elke Stadelmeyer, Thomas Bauernhofer, Wolfgang Schreibmayer.
Medical University Graz, Graz, Austria.

Overexpression of the gene encoding GIRK1 (***G***-protein activated, ***i***nwardly ***r***ectifying ***K***aliumchannel, subunit 1) has been reported to occur in primary invasive breast carcinomas and to correlate with metastasis and prognosis (Stringer et al., 2001). Whether GIRK has a pathophysiological function in the course of cancerogenesis is unknown. Aim of this study was to identify and characterize K+ channels in several breast cancer cell lines (MCF7, MCF10A, MCF12A, MDA453, SKBR3 and T47D). This was done by (i) Western blot analysis, (ii) cloning and expression of cDNA, encoding different GIRK1 splice variants and (iii) functional characterization of K+ channels in the aggressive luminal-type MCF7 and the non-tumourigenic basal B-type MCF10A cell line via the patch clamp method.
Western blot analysis with an antibody directed against the GIRK1 C-terminus identified proteins of different size with differential abundance in the six cell lines. Accordingly, analysis of the RNA isolated from MCF7 cells revealed, that different splice variants, encoding 4 different proteins, occurred. These proteins were expressed in *Xenopus laevis* oocytes and only the full length GIRK1a splice variant was able to form functional K+ channels with either GIRK4 or GIRK2. Single channel analysis revealed completely different K+ channel populations in the plasmamembrane of MCF7 and MCF10A cells. In MCF10A cells a high conductance, depolarization activated, ion channel and a hyperpolarization activated inwardly rectifying potassium channel were found. These channels were never encountered in the MCF7 cell line. Instead, MCF7 cells possessed mechanosensitive, inwardly rectifying cation channels, that were in turn never detected in the MCF10A cell line.
Our study clearly shows that ion channel populations in benign and aggressive breast cancer cell lines differ completely. Future experiments will show whether the different GIRK1 splice variants are related to functional ion channels in these cell lines.

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The Structural Basis for Antidepressants Block Being Confined to Kir4.1

Kazuharu Furutani[1,2], Atsushi Inanobe[1,2], Yoshihisa Kurachi[1,2].
[1]Osaka University, Graduate School of Medicine, Suita, Osaka, Japan, [2]Osaka University, Center for Advanced Medical Engineering and Informatics, Suita, Osaka, Japan.

Subunit-specific ion channel blockers are useful tools for studying physiological functions of the channels. Multiple inwardly rectifying potassium (Kir) channels are differentially distributed throughout the body and play diverse functional roles, but only a limited number of Kir subunit-specific blockers are available. We have found that a series of antidepressants preferentially block Kir4.1 channel over the other Kir subunits, and identified that Thr128 and Glu158 at the Kir4.1 pore are indispensable for binding of blockers to the channel. However, molecular determinants for differential block among Kir channels of antidepressants are still elusive. Here, using an interactive analysis, we address the issue. Fluoxetine and nortriptyline block Kir channels in the rank order of efficacy, Kir4.1 > Kir2.1 >> Kir1.1. Alignment of different Kir subunits shows that threonine at the putative drug interaction site 128 in Kir4.1 is conserved among the other Kir subunits, whereas the amino acid corresponding to Glu158 in Kir4.1 is not conserved and is Asp172 in Kir2.1, and Asn171 in Kir1.1. We demonstrated that it was possible to construct a high-affinity interaction site at position 171 in Kir1.1 by single amino acid substitutions with the same order of efficacy, Glu > Asp > Asn (Kir1.1 wild-type). Therefore, the differential affinity of Kir channels for these drugs is primary due to a single amino acid at this position and these drugs require a negatively charged carboxyl group for high-affinity interaction while the length of the side chain is secondary in the interaction. Conversely, 3D-QSAR model of Kir4.1 blockers-based screening for novel blockers identified some classes of clinically used drugs, which have inhibitory effect on Kir4.1 channel, but negligible effect on Kir1.1 channel. This result supports the feasibility of design of subtype-specific Kir channel blockers despite their highly conserved structure.

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Electrostatic Interactions Between Polyamines And Charged Adducts In The Kir Inner Cavity

Harley T. Kurata, Alejandro Akrouh, Emily A. Zhu, Colin G. Nichols.
Washington University School of Medicine, St. Louis, MO, USA.

Physiological regulation of conductance in Kir channels is accomplished by voltage-dependent block by intracellular cations (Mg2+, polyamines). We have investigated the fine details of polyamine binding in Kir channels, and report unique and unexpected effects of cysteine modification in the inner cavity. Introduction of positive charges near the spermine binding site in Kir6.2[N160D] channels alters the kinetic and steady-state properties of spermine block, although specific effects depend dramatically on both the modified position, and the properties of the modifying agent. MTSEA or MTSET modification of L157C, one helical turn above residue 160, dramatically reduces spermine affinity, and accelerates spermine unbinding. However, effects of MTSEA vs. MTSET modification of L164C, one helical turn below residue 160, are significantly divergent. MTSEA modification again reduces spermine affinity, whereas MTSET has no effect on steady-state spermine block, and slows spermine block and unblock. This stark difference between MTSEA and MTSET is attributed to interactions of the carboxylate sidechain at residue 160 with the primary amine of the ethylamine adduct (MTSEA), which are lost with the quaternarized ethyl-trimethylamine adduct introduced by MTSET modification. Thus, MTSEA modification of L164C reduces spermine block indirectly, by neutralization of the nearby â€~rectification controllerâ€™ residue, rather than a direct interaction with spermine. In contrast, the chemistry of MTSET precludes this interaction, leaving spermine affinity unaltered. Importantly, MTSET modification of L164C reduces affinity for extended spermine analogs, whereas incorporation at a shallower site (S212C) again slows dissociation of extended blockers, shedding light on the localization of the trailing ends of polyamine analogs in the pore. Collectively, the data demonstrate the subtle effects of charge modification in the inner cavity on polyamine-mediated inward rectification, and confirm stable spermine binding between the rectification controller and the selectivity filter.

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Rescue and Gating of a Disease Mutation at an M2 glycine in Kir6.2 of ATP-Sensitive Potassium (KATP) Channels

Jeremy D. Bushman, Paul H. Tewson, Show-Ling Shyng.
Oregon Health & Science University, Portland, OR, USA.

A glycine in the M2 helix of inwardly-rectifying potassium (Kir) channels was hypothesized to bend M2 and gate the intracellular helix-bundle crossing. Bacterial crystal structures position the glycine near the selectivity filter at the extracellular end of the pore. Our previous work characterized a mis-sense mutation at this glycine position identified in a patient with congenital hyperinsulinism (Pinney SE et al., 2008) that would generate Kir6.2 G156R mutant KATP channels. Mutant channels showed near WT surface expression in mammalian cells but no channel activity from inside-out excised patches when heterologously expressed *in vitro*. Further, additional mutations at G156 produce functional channels only if residues are small and uncharged.