The role of G proteins in assembly and function of Kir3 inwardly rectifying potassium channels

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Key words: Kir3 channels, G proteins, trafficking, neurons, cardiomyocytes

Kir3 channels (also known as GIRK channels) are important regulators of electrical excitability in both cardiomyocytes and neurons. Much is known regarding the assembly and function of these channels and the roles that their interacting proteins play in controlling these events. Further, they are one of the best-studied effectors of heterotrimeric G proteins in general and Gβγ subunits in particular. However, our understanding of the roles of multiple Gβγ binding sites on Kir3 channels is still rudimentary. We discuss potential roles for Gβγ in channel assembly and trafficking in addition to their known role in cellular signaling.

Introduction

Kir3 channels are comprised of four different but homologous subunits, Kir3.1-3.4, which are conserved in mouse, rat and human, each capable of playing a unique functional role in channel activity, with distinct regulatory features, tissue and subcellular distributions. Our focus here will be on the roles of heterotrimeric G proteins and other interacting proteins in channel assembly, trafficking and function. Interested readers are directed to more comprehensive recent reviews on other aspects of channel function.1-3

Cloning and Distribution of Kir3 Channels

The primary sequences of the first inwardly rectifying K+ (Kir) channels were elucidated in 1993. cDNAs of two Kir channels, ROMK1 (renal outer medulla K+ channel), an ATP-dependent Kir channel, and IRK1 (inward rectifier K+ channel), were isolated by expression cloning from the outer medulla of the rat kidney4 and a mouse macrophage cell line, 5 respectively. Following identification of ROMK1 and IRK1, a number of other Kir channel subunits were also cloned. Now 15 distinct Kir channels have been cloned which are divided into seven subfamilies (reviewed in ref. 1). Of particular interest here are the Kir3 channels of which there are four mammalian subunits, Kir3.1, Kir3.2, Kir3.3 and Kir3.4, which exhibit a similar primary structure (Fig. 1).

Kir3 channels are widely expressed in most mammalian species including the human, rat and mouse.1,6 All four subunits are well conserved between species with Kir3.1-Kir3.3 subunits having ~98–99% and Kir3.4 having 94% sequence identity. northern blot analysis of human peripheral and brain tissue,7 indicated that Kir3.1 is expressed in kidney, heart and brain tissue with the highest levels found in the latter, specifically the amygdala and hippocampus. Kir3.3 was determined to be highly expressed in all of the examined brain areas, while Kir3.4 was primarily expressed in the pancreas and to a lesser degree in the heart, placenta, lung and kidney with negligible expression in the brain. Kir3.2, of which there are four splice variants, was found to be expressed minimally in the periphery but at high levels in the brain, particularly in the substantia nigra, amygdala and hippocampus,7 where the Kir3.2c variant is most abundant.8,9

Recent studies have shown that distinct populations of Kir3 channels can be detected in different cell populations of mouse cerebellum suggesting that their synthesis, trafficking and molecular organization into signaling complexes are tightly regulated and highly dynamic processes.10 Unique phenotypes associated with deletion of Kir3.1,11,12 Kir3.2,12-17 Kir3.3,14,15,17,18 and Kir3.419-21 have been described as well (reviewed in ref. 22).

Structure of the Kir3 Channel

The four cloned human Kir3 subunits encode proteins composed of anywhere from 393 to 501 amino acids, which share approximately 36% sequence identity (reviewed in ref. 23). Two-thirds of the amino acid sequence of each Kir subunit is characterized by large hydrophilic amino- and carboxy-terminal domains that extend from 90 to over 200 amino acids into the cytoplasm, respectively. These intracellular domains, which also include the cytoplasmic pore region, are of crucial importance for channel modulation by many intracellular regulators, but
and ascending pore helices flank the well-conserved potassium channel signature sequence, TXGYG or TXGFG.25 Point mutations within this sequence abolish potassium selectivity and thus, for this reason, the pore loop is thought to form the selectivity filter of the channel.30 A topographical view of the cytoplasmic pore of the channel obtained from a high-resolution structure of the Kir3.1 channel subunit showed that the pore measures ~30 Å in also for establishing the strong voltage dependence of inward rectification.24 In the transmembrane domains and the pore regions of the channels however, the level of sequence identity increases to ~80–90%. The Kir3.1 subunit is the largest subunit in the Kir3 family, and also one of the most divergent of the four since it shares only 44% sequence identity with the other three subunits. Kir3.2, Kir3.3 and Kir3.4 are more closely related, sharing 62% sequence identity.

Each subunit of the tetrameric Kir3 channel is made up of two transmembrane-spanning helices which flank a highly conserved pore-forming helix.25,26 The high-resolution crystal structures of KcsA and KirBac1.1, two bacterial homologues of potassium channels closely related to eukaryotic Kir channels, confirmed many of the architectural features of the Kir channel family.27,28 In the KcsA and KirBac structures, the outer transmembrane helix (M1) has been shown to contact the inner transmembrane helix (M2), which lines the pore of the channel.26 The pore loop, which spans a length of ~100 amino acids, is located between the M1 and M2 helices.24,27,29 Descending
Modulation of Kir3 Channel Activity

The biophysical fingerprint of Kir3 channels is their characteristic inward rectification, which depends critically on positively charged polyamines that reside in the cytoplasmic side of the cell. Under experimental conditions, this inward rectification can give rise to large inward currents at hyperpolarized potentials and small outward currents under depolarized potentials. The limited potassium flux at depolarized potentials is due to the blockade by intracellular magnesium and polyamines such as spermine and spermidine. Thus, unlike the family of voltage-gated potassium channels, the Kir3 channels structurally lack an intrinsic voltage sensor segment, despite the fact that their activity is voltage-dependent.

Kir3 channel gating is dependent on the presence of membrane phosphatidylinositol 4,5 bisphosphate (PIP₂) levels. As such, Kir3 channel currents are enhanced in the presence of PIP₂ and its depletion usually leads to channel rundown. Recent studies have focused on localizing the N- and C-termini of the Kir3 channel (reviewed in refs. 6 and 35) and recent studies have shown that mutations on either termini reduce PIP₂ binding. One of these residues is a conserved basic arginine residue which according to the KirBac structure, lies in the M2 segment of the channel, just below the membrane.

The channel-PIP₂ interaction of the Kir3 channels is sensitive to regulation by various modulators such as sodium, intracellular pH, arachidonic acid and G proteins (reviewed in ref. 33).

Gβγ subunits. In addition to polyamines and PIP₂, heterotrimeric Gβγ subunits also play a crucial role in modulating Kir3 channel activity. Some of the earliest work on Kir3 channels, given that they underlie the atrial muscarinic acetylcholine potassium channel, was performed in the Vagus system in cardiomyocytes. Although it was clear for many years that the Kir3 channels were coupled to and activated by pertussis toxin-sensitive GPCRs such as the muscarinic M2, δ-opioid, dopamine D₂ or somatostatin receptors, the G protein subunit directly responsible for channel activation was initially controversial, but there are now numerous lines of evidence that support the notion of Gβγ dimers as central activators of these channels. For instance, purified Gβγ subunits from the bovine brain have been shown to activate Kir3 channels. This finding was later confirmed in heterologous expression systems as well as in excised patches from atrial myocytes (reviewed in refs. 6 and 41). Overexpression of Gβγ subunits in Xenopus oocytes was also shown to increase basal Kir3 channel currents. Additionally, Gβγ sequestration through phosducin or Gβγ-binding peptides has been shown to render the channel inactive, even when stimulated by neurotransmitters, thereby reinforcing the role of the Gβγ subunits in Kir3 channel activation.

Using channel fusion proteins, a number of studies have demonstrated the direct binding of Gβγ subunits to the full-length Kir3 channel or to segments of the intracellular N- and C-terminal domains of the channel (reviewed in refs. 1, 6 and 41). Recent mutagenesis work has also confirmed the contribution of the Gβγ subunits in Kir3 channel regulation. Specific point mutations in the Gβ subunit have been shown to block the ability of the G protein to regulate Kir3 channel activity. Likewise, mutations in the C-terminal extremity of the Gγ₂ subunit, demonstrated that this subunit was required for the activation of the channel.

Electrophysiological studies have shown that deletion of the Gβγ binding site on the N-terminus of Kir3.1 is associated with the loss of the fast activation and deactivation kinetics which usually accompanies Kir3 channel activation (reviewed in ref. 6). Similarly, peptides derived from the N- and C-termini of the Kir3 channel were shown to block G protein modulation of the channel in excised patches. Co-immunoprecipitation of Kir3.1 and Kir3.4-containing subunits and Gβγ from atrial membranes with antibodies against Kir3.1 and Kir3.4, have also supported the idea of an interaction of the G protein with the channel. Thus, direct interactions between the Gβγ subunits and the channel are required for channel activation.

Much work has also focused on precisely localizing the interaction sites of Gβγ with Kir3 channels. For example, mutagenesis has helped identify critical regions of the channel important for Gβγ binding. Mutation of a histidine-64 residue in the N-terminus of Kir3.4 and a leucine-268 residue in the C-terminus of Kir3.4 have shown that they are critical for Gβγ-mediated channel activity. Similar mutations in the Kir3.1 channel have also helped identify residues which regulate the Gβγ-Kir3.1 channel interaction. The reverse approach of using Gβ mutations has also aided in the discovery of functionally important channel interaction sites with Gβγ. For example, mutations at threonine-86, threonine-87 and glycine-131, all located on the outer loops of the Gβ, subunit, were shown to substantially reduce Kir3 channel activation, suggesting that these residues lie in the Gβ-Kir3 channel interface.

X-ray crystallography has revealed that the Gβ protein has the structure of a seven-bladed propeller with four anti-parallel β strands per blade. The Go subunit has been shown to interact with the Gβ subunit through the top of the propeller, while the Gy subunit appears to interact with the Gβ subunit through the bottom of the propeller. Substitution of alanine for tryptophan-332 on the Gα/Gβ-interacting surface impaired the interaction between Gβ and the Kir3 channel. However, Gβ surfaces which lie outside the Gα/Gβ-interface may also contain Gβ-channel interaction sites. Additionally, distinct effector binding domains within Gβγ may differentially regulate effector functions since mutations of Gβγ have been shown to alter the regulation of specific effectors without affecting other Gβγ-dependent functions.
Gα subunits can also interact directly with the intracellular domains of the Kir3 channels. In vitro binding studies have identified six amino acids in the Kir3 C-terminal domain which are essential for Gαi binding.38,60,62 Gα subunits have been shown to determine the specificity of G protein action.32,38,60,66 For example, chimeric studies have revealed the Gαi subunit, but not the Gαs or Gαq subunits, as critical determinants of Gβγ specificity for Kir3 channel modulation.32 Thus, Kir3 channel activity is dependent on the specific Gα subunit to which the Gβγ subunits are associated.32,38 Recent studies have also demonstrated using a combination of in vitro protein/protein interaction assays, BRET, FRET and total internal reflection fluorescence microscopy (TIRF) that the G protein heterotrimer remains associated with Kir3 channels throughout the cycle of channel activation.38,65,66 Taken together, there is data suggesting that Kir3 channels interact with G protein heterotrimers.

Gαq subunits can also interact with the Kir3 channels. Pull-down assays have shown that it is actually the N- rather than the C-termini of the Kir3.1, Kir3.2 and Kir3.4 channels which interact with the Gαq subunit. Gαq likely binds between residues 51 to 90 on Kir3.2. The functional significance of the Gαq-channel interaction might be explained by the actions of signaling pathways downstream of Gαq which inhibit Kir3 channel activity, i.e., the regulation of PIP levels (discussed above). Recent studies have also shown that combinations of heterotrimeric G proteins also interact with distinct Kir3 isoforms. For example, Kir3.1-containing channels interact with intact Gαq, heterotrimers via their unique C-terminal domain in a manner that Kir3.2 homotetramers channels do not, yielding a unique pattern of regulation for Kir3.1.64

In native tissues, only Gαi-coupled, but not Gαs- or Gαq-coupled receptors, seem to activate Kir3 channels despite the fact that: (1) the latter G proteins theoretically liberate Gβγ upon receptor stimulation, and (2) coupling of receptors to Kir3 channels can be induced by overexpression of Gαs.58,67 How then, is receptor and G protein specificity achieved? Since a number of Gβγ subunit combinations have been shown to activate the channel, it is unlikely that receptor selectivity lies at the level of the Gβγ dimers. A recent study suggests that certain combinations of G proteins, such as heterotrimeric Gs, may be spatially restricted from interacting with Kir3 channel complexes by differential distribution into distinct lipid microdomains.70

Other Gα subunits may play key roles in determining the specificity of coupling between receptors and the channels. A recent study showed that swapping the receptor coupling specificity of the Gαi and Gαq subunits did not compromise G protein effector specificity.63 However, chimeras between the Gα
and G_{q} subunits have revealed that the helical domain of G_{q}\textsuperscript{i} (residues 63–175) is a critical determinant of G_{q}\textsubscript{b\gamma} signaling specificity. Thus, G_{q}\textsubscript{b\gamma} specificity is dependent on the specific G_{q} subunit with which it is associated. Chimeric studies have also been conducted with the G_{q}\textsuperscript{i} and G_{q}\textsubscript{o} subunits and support the central role of the G_{q} subunits in determining receptor-coupling specificity to Kir3 channels. These findings are exemplified by the fact that the overexpression of G_{q}\textsubscript{o} or G_{q}\textsubscript{b\gamma} proteins does not enhance channel activation.\textsuperscript{63,64} As mentioned above, there is preferential binding of PTX-sensitive G_{q}\textsuperscript{i} subunits to the C-terminus of Kir3.2 while PTX-insensitive G_{q}\textsubscript{o} and G_{q}\textsubscript{b\gamma} proteins do not interact with the C-terminus, suggesting that the G_{q}/C-terminus interaction may regulate receptor coupling to Kir3.2 channels.\textsuperscript{38}

RGS proteins and Kir3 channels. “Regulators of G protein signaling” (RGS proteins) play a crucial role in G protein-mediated regulation of the Kir3 channels. There are more than 30 RGS proteins coded by the mammalian genome, many of which affect specific GPCR signaling pathways (reviewed in ref. 71). Classically, RGS proteins are GTPase activating proteins which accelerate the intrinsic GTP hydrolysis mediated by the G_{q} subunit but have a number of functions unrelated to their GAP activity.\textsuperscript{72} RGS proteins functionally accelerate both Kir3 channel activation and deactivation kinetics by up to 100-fold.\textsuperscript{73-75} Kinetic experiments aimed at deciphering the role of RGS proteins have demonstrated that RGS1, RGS3 and RGS4 significantly increased the rate of Kir3.1/3.2 channel activation and deactivation in response to receptor stimulation. Other studies in various heterologous expression systems have established that the Kir3 channels can also be modulated by a number of other RGS proteins, suggesting little RGS protein specificity for effects on Kir3 channels.\textsuperscript{76} The RGS core domain of RGS8 has been implicated in accelerating the kinetics of deactivation, while the N-terminal domain appears to mediate the acceleration of the activation kinetics of the channel.\textsuperscript{77}

No studies have reported a decrease in the amplitude of GPCR-induced currents in the presence of RGS proteins. RGS proteins certainly increase the rate of GTP hydrolysis, resulting in an increase in the concentration of Go\textsubscript{GDP} which in turn, sequester activated G_{q}\textsubscript{b\gamma}, thus accelerating the deactivation kinetics of the channel.\textsuperscript{78} How channel activation kinetics increase is less clear. This might be explained by functional or structural changes in the heterotrimeric G proteins associated with Kir3-based signaling complexes. Thus, they modulate the activity of the complex as a whole. It is clear that several RGS proteins interact with many of the components of GPCR signaling pathways including Kir3 channels, and not simply G_{q} subunits (reviewed in refs. 2 and 78).

FRET and co-immunoprecipitation experiments have shown that RGS4 can stably associate with several signaling proteins to form a complex involving the GABA\textsubscript{A} receptors, G_{x}\textsubscript{o} and Kir3 channels.\textsuperscript{79,80} These findings provide a novel model of action for the RGS proteins in which they can enhance the kinetic efficacy of the channel by their direct association with the G_{x} subunit. In addition, these findings also support the idea that RGS proteins are accessory proteins, which form a part of larger signaling complexes.

### Trafficking of Kir3 Channel Complexes

Regulation of Kir3 trafficking provides a potential mechanism to regulate cellular excitability. Though much is known regarding Kir3 trafficking as it progresses along the biosynthetic pathway, fewer studies have attempted to characterize trafficking itineraries once internalized from the plasma membrane. In this section we will discuss anterograde trafficking of Kir3 channels as they exit the endoplasmic reticulum (ER) as well as the life cycle of a Kir3 tetramer once it forms a functional channel at the cell surface.

Kir3.1 lacks an ER export signal and therefore is retained in the ER unless co-expressed with another Kir3 subunit (reviewed in refs. 81 and 82). Studies of the assembly of the eponymous I_{K\textsubscript{ACH}}, a heterotetramer composed of Kir3.1 and Kir3.4 subunits,\textsuperscript{83} have demonstrated that Kir3.4 is required for the maturation and surface delivery of Kir3.1 in COS-7 cells.\textsuperscript{84} When expressed alone, recombinant Kir3.1, which contains only one potential site for N-linked glycosylation, migrates as a doublet with a molecular mass of 54 and 56 kDa, with the upper band being the core-glycosylated, immature form of the protein.\textsuperscript{83,84} Upon treatment with either endoglycosidase H, an enzyme that selectively removes N-linked glycosyl moieties from proteins that have not been processed in the Golgi or endoglycosidase F, an enzyme that non-selectively removes all N-linked sugar residues, the 56 kDa band is virtually abolished, confirming ER residence. Interestingly, after co-expression of Kir3.4 with Kir3.1, a unique band was observed at a molecular weight above the immature Kir3.1 doublet which represents fully mature, properly glycosylated Kir3.1.

Further evidence that Kir3.4 plays an important role in Kir3.1 processing and trafficking comes from confocal experiments using extracellularly Flag-tagged Kir3.1 subunits that were only detected at the cell surface upon co-expression of Kir3.4 in non-permeabilized cells.\textsuperscript{81} When expressed alone, Kir3.1 channels remain in the ER, associated with intermediate filaments of the cytoskeleton. Though it is known that an ER export signal is present on the N-terminus of Kir3.4,\textsuperscript{81} it was of interest to determine which precise region(s) of Kir3.4 are involved in Kir3.1 trafficking. When chimeras containing the pore and C-terminus of Kir3.1 were fused to the N-terminus of Kir3.4, no functional channel could be detected. When chimeras containing the C-terminus of Kir3.4 was expressed, it was still unable to be delivered to the surface in COS-7 cells suggesting multiple determinants are involved in channel trafficking.\textsuperscript{84} Deletion analysis indicated different regions of the Kir3.4 C-terminal domain were important for surface delivery of Kir3.4 homotetramers compared to Kir3.4/Kir3.1 heterotetramers. Specifically, amino acids 375–399 were essential for heterotetramer surface localization, whereas amino acids 350–374 were critical for homotetramer surface localization. Similar studies using atrial myocytes from Kir3.4

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knockout mice corroborate the dependence of Kir3.1 maturation and surface delivery on the presence of Kir3.4.19

Kir3.2 subunits have been found to play a similar vital role in forming functional Kir3.1-containing channels at the cell surface. Kir3.2 subunits form both homotetramers as well as heterotetramers with either Kir3.1 or Kir3.3 which can then be targeted to the plasma membrane.85 Deletion analysis again identified ER export signals found at both the N- and C-termini of Kir3.2, vital for cell surface delivery of Kir3.1/3.2 heterotetramers.81 Though no well defined ER export motif has been characterized for Kir3.3, it has been shown to form tetrameric channels with both Kir3.1 and Kir3.2 subunits.86 Kir3.3 function in channel trafficking has received little attention, however the presence of a C-terminal lysosomal-targeting motif has led some to suggest a role for Kir3.3 in the reduction and degradation of Kir3 channels from the cell surface, thereby adding another layer of regulation to cellular excitability.81,82 In fact, when Kir3.3 subunits were co-transfected with Kir3.1 and either Kir3.2 or Kir3.4, a significant reduction in cell surface expression of all Kir3.1-containing channels was observed.81 However, there is evidence that channels containing mouse Kir3.3 might be functional when complexed with Kir3.1.86 Thus the roles of individual combinations of channel subunits may be species- and/or cell type-specific. Distinct cell types in the cerebellum, for example, show striking differences in the distribution of Kir3.1, Kir3.2 and Kir3.3.10 This cell specificity may depend on differential distribution of channel interacting proteins as well.

Interacting Proteins that Regulate Kir3 Channel Assembly or Trafficking

During protein biosynthesis, membrane proteins are transported to the cell surface via trafficking pathways dependent on Sar or Rab GTPases, which govern distinct steps of both the exocytic and endocytic pathways.86,87 For example, Rab1 regulates ER-Golgi transport, whereas Rab6 regulates intra-Golgi transport.88 We used dominant negative (DN) mutants of the small G proteins, Sar1 and various compartment-specific Rabs, which impede anterograde protein trafficking at different steps. Sar1 H79G and Rab1 S25N mutants efficiently blocked the plasma membrane trafficking of the Kir3.1/Kir3.4 complex suggesting that they are trafficked via these conventional pathways.89 Later, we discuss where the first interactions of Kir3 channels with GPCRs and G proteins occur during channel trafficking.

Another potential mechanism whereby Kir3 channel surface expression could be enhanced in endocrine cells was demonstrated by antibody labelling Kir3 channels localized in dense core vesicles within endocrine cells of the anterior pituitary lobe.90 These Kir3 positive vesicles also contained thyroid-stimulating hormone (TSH), which upon stimulation by thyrotropin-releasing hormone (TRH), fused to the plasma membrane incorporating Kir3 channels into the cell membrane while simultaneously secreting TSH. Since only Kir3.1 and Kir3.4 mRNA have been detected in the anterior pituitary, it was concluded that these channels were likely heterotetramers of Kir3.1/3.4. The authors confirmed the functional surface expression of these Kir3 channels by demonstrating that upon TRH stimulation, addition of dopamine or somatostatin, significantly increased the inwardly rectifying current characteristic of Kir3 channels. The above experiments provide a novel means for endocrine cells to downregulate their excitability after stimulation by TRH through a negative feedback mechanism mediated by the fusion of vesicles containing both TSH and Kir3 channels.

Once Kir3 channels have incorporated themselves into the surface membrane, they may adopt alternate fates depending on their subunit composition. Kir3.2a-containing channels are unique in so far as they have a N-terminal “VL” internalization motif as well as a C-terminal cluster of acidic amino acids which have been shown to mediate much of their post-internalization trafficking.81 When Kir3.2a channels were expressed in COS7 cells either with or without Kir3.1, immunofluorescence experiments showed that many channels could be found in late endosomes/lysosomes as denoted by colocalization with GFP-Rab7.81 When this VL motif was mutated to alanine, late endosome colocalization was drastically reduced and a much larger fraction of the channels could be found at the surface. This suggested that the VL internalization motif was essential in regulating the number of Kir3.2a containing channels at the cell surface.

A number of other proteins have also been demonstrated to regulate levels of Kir3 channels. Mice with the gene for neural cell adhesion molecule (NCAM) deleted showed altered responses to 5-HT1A receptor agonists, with stimulated Kir3 currents drastically increased by over 200% in hippocampal slices.90 Both NCAM140 and NCAM180 are transmembrane proteins associated with lipid rafts, while NCAM120, another family member which contains no intracellular domains is targeted to the plasma membrane via a GPI linker.92 When NCAM140 or 180 were co-expressed with Kir3.1/3.2 and 5-HT1A in Xenopus oocytes (and CHO cells), a striking reduction in Kir3 current amplitude and cell surface channel expression was observed relative to either control or NCAM120-expressing cells. Kir3.1/3.3 channels expressed in the same context also showed a decrease in Kir3 current, yet Kir3.1/3.4-containing cells surprisingly showed no differences in current amplitude when co-expressed with NCAM 180, lending support to the notion that NCAM specifically regulates neuronal Kir3.2 and Kir3.3 channels.91 Hybrid channels, where the N-terminus of Kir3.2 was switched for the N-terminus of Kir3.4 (or vice versa), resulted in Kir3.1/3.4 currents sensitive to expression of NCAM180 while Kir3.1/3.2 channels lost their sensitivity to NCAM. Thus, the N-terminus of Kir3.2 conferred the necessary structural elements for NCAM regulation. More recently, the same group demonstrated that NCAM and the neurotrophin receptor TrkB competed for the binding site on the C-terminal domain of Kir3.3 but not Kir3.2.92 Interestingly, TrkB binding increased the surface expression of Kir3.3 in contrast to the effect of NCAM. These studies again highlight effects of distinct sets of interacting partners on Kir3 isoforms. It was shown using cell-surface biotinylation that NCAM did not influence internalization of Kir3 channels from the plasma membrane. Further, the channel was not retained in the ER and likely NCAM asserted its control on Kir3 somewhere in the Golgi network. Finally, the effects of NCAM on Kir3 trafficking were shown to be lipid-raft dependent.93
Kir3.2c as well as Kir3.3 possess PDZ binding motifs at their C-termini. Although many neuronal proteins contain PDZ domains, few have been shown to interact with either subunit. For example, neither Kir3.2c or Kir3.3 are able to interact strongly with canonical PDZ domain-containing proteins such as PSD-95 and Chapsyn-110, even though the related Kir2.1 subunit, which also contains a PDZ ligand on its C-terminal tail, was found to bind to these proteins. The difference between the PDZ ligand of Kir2.1 and Kir3.2c/Kir3.3 is rather subtle, with the former being ESEI and the latter ESKV. Using a proteomic approach, a recent study identified a novel Kir3 regulatory protein, sorting nexin 27 (SNX27), which contains a PDZ domain that could bind both Kir3.2c and Kir3.3, but not Kir2.1. SNX27, which is a member of the sorting nexin family involved in intracellular sorting of proteins from various endosomal compartments, also contains a PI3P-binding PX domain and a Ras-associated domain. As PI3P is known to be enriched in early endosomes, immunofluorescence was used to demonstrate that SNX27 colocalized with early endosome antigen 1 (EEA1), primarily found in early stages of the endocytic pathway. In order to determine the role of SNX27 in surface expression and trafficking of Kir3 channels containing PDZ binding motifs, patch clamp experiments were carried out using HEK293 cells transiently transfected with Kir3.1/Kir3.3, the GABAβ receptor and SNX27. Interestingly, a reduction in current as well as reduced Kir3.1 protein levels were observed in cells co-expressing SNX27, suggesting that it aids in removal or degradation of Kir3.1/Kir3.3 containing channels.

To evaluate the relevance (or apparent redundancy) of the PDZ binding motif of Kir3.3 and its functionally similar lysosomal targeting sequence, the final four amino acids of Kir3.3 were removed (i.e., the PDZ binding domain). When GABAβ-induced currents were measured, a striking decrease was noted in baclofen-induced potassium currents, suggesting that it aids in removal or degradation of Kir3.1/Kir3.3 containing channels.94 Kir3 internalization from the plasma membrane has received less attention to date than anterograde trafficking. Though they were initially believed to be endocytosed via a clathrin-mediated process, akin to many GPCRs to which they are coupled, a growing amount of evidence suggests alternative routes for channel internalization. One such line of evidence comes from the observation that Kir3.4 has a cluster of acidic C-terminal amino acids akin to certain splice variants of Kir3.2. A significant reduction in Kir3 surface expression was noted when the acidic cluster was mutated, thus this may be a bona fide surface trafficking motif. In both primary hippocampal neurons and mouse HL-1 cells, wild-type Kir3.4 was internalized through a clathrin-independent process that involved an initial association with Arf6-positive endosomes. An alternative pathway for Kir3.4 internalization is macropinocytosis, which has also been shown to involve Arf6, and is readily induced via PMA activation of PKC. Upon stimulation of COS7 cells and primary hippocampal neurons with PMA, a drastic reduction of Kir3.4 surface expression was noted accompanied by the presence of large endocytic vesicles containing Kir3.4 channels. This was effectively blocked by inhibitors of PKC as well as known macropinocytosis blockers, such as amiloride and wortmannin, but not by clathrin inhibitors such as AP180C and Eps15(Δ92/295).

**Larger Signaling Complexes Containing Kir3 Channels**

Functions for Gβγ beyond opening Kir3 channels. A recent mathematical model integrates many of the functional and structural studies highlighting the roles that Gβγ and RGS proteins...
play in modulating channel function. However, a number of studies have demonstrated that Kir3.1 channels, expressed alone, can interact with heterotrimeric G proteins. Studies of the functional stoichiometry of Gβγ with respect to channel activation indicate that up to four Gβγ subunits can bind which would lead to maximal channel opening. In addition, cross-linking experiments have suggested the binding of four Gβγ subunits per tetramer, attributing one Gβγ binding site per channel subunit (reviewed in ref. 41). Electrophysiological analyses have indicated that varying the number of Gβγ subunits bound to channel tetramers can modulate the extent of channel activation. Gβγ binding, especially when all potential sites on the channel are occupied, may also alter the properties of the pore such that Kir3 channels are less sensitive to block by extracellular ions such as Cs and Ba.

Direct activation of the Kir3 channels by Gβγ is mostly independent of the composition of the heterodimer, suggesting that there is little specificity for Gβγ subunit modulation of channel activity. However, it is possible that G protein specificity, whose impacts on signaling may not be manifest when studying the channel opening event in isolation. For example, it has been demonstrated that signaling specificity between Kir3 and Gβγ may depend on other components of the larger complex containing the GPCR as well. Beyond the need for maximum binding of four Gβγ subunits to Kir3 channels, why are there distinct classes of sites for Gβγ and Gα binding on the N- and C-termini of Kir3 channels? Until recently, the specificity of precocious (i.e., agonist-independent interactions which may occur during Kir3.1 and Kir3.2 channel trafficking or assembly) interactions between different Gβγ subunit combinations and the Kir3 channels had not been addressed. We demonstrated that there was in fact G protein specificity in the early channel-G protein interactions inside the cell. For example, Gβ1-4-containing Gβγ dimers could interact with the Kir3.1 channel, although through different relative G protein-channel affinities. In contrast, Gβ5 did not interact with the channel in the ER. Kir3.2 on the other hand, only interacted with Gβ1- and Gβ2-containing dimers, but not Gβ3-5 in the absence of agonist stimulation. In both cases, although Gβ5 could not interact directly with the channel, it could be detected in larger complexes containing the channel and may require RGS proteins for functional effects on the channel.

The roles of the numerous putative Gβγ binding sites on Kir3 channel tetramers are still unclear. Previous studies have indicated that the binding of Gβγ subunits to only a subset of Kir3 channel binding sites is sufficient to fully open the channel, suggesting that these sites may be required for Gβγ-dependent signaling events which occur at the cell surface following receptor activation. These observations raise the question of what functional role the other Gβγ binding sites on the channel could serve. Mutations within the Gβγ-binding segment of the C-termini have been shown to eliminate agonist-induced receptor activation of the channel but preserve the Gβγ-dependent basal activity, implying that the latter is controlled by a separate binding site on the channel. Therefore, one hypothesis is that some Gβγ binding sites are required for the early interactions between the G protein and the channel during protein biosynthesis and the assembly of G protein-channel complexes, whereas other sites may be required to mediate channel activation at the cell surface. Deciphering the roles of the individual Gβγ binding sites on the Kir3 channel and understanding which sites play a role in the early or precocious interactions compared to the signaling interactions will be of critical importance in order to model the molecular mechanisms of the G protein-Kir3 channel interaction.

### Organization of Kir3 Channel Complexes

Although the Kir3 channels are activated by Gβγ subunits, their function can also be modulated by a large number of signaling molecules, raising the possibility that some or all of these molecules might be organized into a macromolecular signaling complex. In recent years, our understanding of G protein signaling suggests that a spectrum of organizational arrangements are used by different signaling pathways, ranging from a series of transient interactions between partners such as in the mammalian visual system to more stably assembled signalosomes that are built and trafficked to the plasma membrane or other subcellular destinations (reviewed in refs. 57 and 109). One end of the spectrum is required where signal amplification is important and specificity is of lesser concern while the other end of the spectrum reflects a need for rapid and highly specific signaling events. The dynamic nature of these complexes would perhaps be better described by the term “meta-stable” wherein individual interactions could be quite labile but many of them together contribute to the overall integrity of the larger complex. This is consistent with the dynamic nature of hydrogen bonding patterns characterizing the Kir3/Gβγ interaction.

A number of GPCRs have been detected as part of macromolecular complexes, which contain Kir3 channels. For example, β2-adrenergic receptors can stably associate with the Kir3.1/3.4 and Kir3.1/3.2 channels as measured using BRET and co-immunoprecipitation. Data from this article also indicated that dopamine D4 receptors associated with the Kir3 channels to form a stable complex. These receptor-Kir3 channel interactions are stable even in the presence of an agonist suggesting that these complexes remain intact throughout the signaling process. The kinetics of signaling through Kir3 channels is also consistent with the notion of stable complexes.

BRET studies and confocal microscopy have given some insight into the steps involved in the formation of these protein complexes and their intracellular sites of assembly. By using dominant negative Rab and Sar1 GTPases, it has been shown that receptor-receptor interactions and receptor-Gβγ interactions initially occur in the ER, after which they can be trafficked out towards the plasma membrane. Similar results have also been observed between the GABA_B receptor subunit and the Kir3.2 channels, where initial interactions occur shortly after protein biosynthesis, followed by anterograde trafficking events. Importantly, a recent study has demonstrated that these GABA_B/Kir3 complexes (including Kir3.1, Kir3.2 and Kir3.3) can be detected in situ in cerebellar Purkinje cells. RGS proteins have also been detected as parts of these complexes.
Co-immunoprecipitation studies have demonstrated that other signaling proteins such as G protein-coupled receptor kinases (GRKs), protein kinases (PKA, PKC) and protein phosphatases (PP1 and PP2A) can also interact with native neuronal and atrial Kir3 channels. Some studies have shown that tyrosine phosphorylation of the Kir3 channel can suppress channel conductance and accelerate channel deactivation, suggesting that tyrosine phosphorylation of the Kir3 channel inhibits channel activity. However, although the mechanism of channel inhibition has been linked to the activation of the Gqα-coupled receptors, there has been much debate as to whether it is really the PKC-mediated phosphorylation of the channel or the phospholipase C (PLC)-mediated PI3 depletion, which ultimately directs channel inhibition. Functional studies have reported the inhibition of Kir3 channels after PKC activation, whereas preventing PLC activation preserves channel activity. One hypothesis is that Kir3 channel inhibition is initially mediated by the depletion of PI3, driven by Gqα-mediated stimulation of PLC. However, the simultaneous increases in diacylglycerol (DAG) levels, resulting from PLC stimulation, could also induce activation of PKC. By subsequently phosphorylating the channel, PKC may further decrease the interaction between the channel and PI3. This either changes the affinity of the channel for PI3, or causes a conformational change in the channel as a result of phosphorylation.

What’s Next?

Several questions arise from these observations. Are interactions between GPCRs and Kir3 channels direct or indirect? Kir3 channels interact directly with G protein heterotrimers. Many GPCRs have also been shown to interact directly with both the Gα and Gβγ subunits. Thus, since both the receptor and the Kir3 channel interact with Gα and Gβγ subunits, whether or not the receptor is activated, receptors and heterotrimeric G proteins could remain associated with the Kir3 channels throughout a basic signaling event. What is the nature of the distinct receptor and channel based complexes? They may be distinct for different receptor homo- or hetero-oligomers or channel homo- and heterotetramers. It is clear that distinct receptor/receptor interactions in the context of GPCR heterodimers can alter the trafficking itineraries of Kir3 channels. For example, a recent study has demonstrated that a complex containing M2 muscarinic receptors heterodimerized with GABA A β2 subunits alters the internalization pattern of M2 receptor/Kir3 channel complexes in PC12 cells, providing another means of regulating cell excitability.

It is clear that Gβγ subunits serve much broader roles in GPCR signaling than simply acting as signaling mediators activated by receptors. They may have roles prior to signaling in the assembly of GPCR signaling complexes (reviewed in ref. 57). What are the roles of the distinct G protein binding sites (between 8–12/ tetramer) on the different Kir3 channel subunits? Can we differentiate between the roles of the Gβγ binding sites on the N- and the C-termini of Kir3 channels by studying channels in which either the N- or the C-terminal Gβγ binding sites are removed? Are Gβγ binding sites on one domain of the channel important for assembly and trafficking events, what we have called “pre-cious” interactions, while Gβγ binding sites on the other domain are critical for the functional regulation of the channel?

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the Heart and Stroke Foundation of Quebec to Terence E. Hébert. Terence E. Hébert is a Chercheur National of the Fonds de la Recherche en Santé du Québec and Peter Zylbergold holds a studentship from the CIHR Drug Discovery Training Program at McGill University.

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