Membrane-delimited modulation of ion channel activity

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Ion channels are transmembrane proteins that conduct ions across cellular membranes. The channel proteins assume a number of conformations some of which are permissive to ion flow and some of which are not. Occlusions of ion flow along the permeation pathway are made by parts of the channel protein referred to as gates and their movement out of or in the way, to allow ion flow or not, is referred to as gating. Gating transitions between states that are permissive [open state(s)] and those that are not permissive [closed state(s)] are controlled by different stimuli, such as voltage across the membrane, ligands acting on either side of the membrane or mechanical deformations of the membrane. A number of additional signals can act on channel proteins to modulate gating and thus channel activity. Here, I focus on signals limited to the plasma membrane (membrane delimited) and using the G protein-gated K^+ channels as the example, I review two major such mechanisms that modulate K^+ ion flow through these channels: through interactions of the channels with G proteins (protein-protein) and with phosphoinositides (lipid-protein). Interactions that stabilize open conformations enhance activity, while those that stabilize closed conformations inhibit activity. A number of channel structural elements (e.g. the permeation pathway with its gates) exhibit a high degree of conservation in the ion channel superfamily, arguing for similarities in the overall design for channel gating. Thus the principles learned by studying this system could be applied to other systems involving similar players or underlying structural elements.

Introduction

The Logothetis lab has focused its efforts over the last couple decades studying modulation of channel activity by membrane-delimited signaling mechanisms. Specifically, modulation of K^+ channel activity by G proteins through direct protein-protein interactions and by phosphoinositides by direct lipid-protein interactions has been the focus of these studies. The players in these two signaling mechanisms, namely the G protein subunits, the phosphoinositides and the K^+ channel they both modulate are introduced below.

The channel: The G protein-sensitive K^+ channels belong to the 15-member family of inwardly rectifying potassium (Kir) channels, so called as they conduct K^+ ions better in the inward ($V_m < E_K$) rather than the outward ($V_m > E_K$) direction (Fig. 1B) (Hibino et al., 2010). Their popular name is GIRK (G protein-sensitive inwardly rectifying K^+) channels, while their official name is Kir3 channels. Four mammalian isoforms comprise the Kir3 subfamily: GIRK1-GIRK4 (Kir3.1-Kir3.4). GIRK1 channels function only as heteromers in association with one of the other subunits. The other subunits (GIRK2-GIRK4) can function both as homotetramers and heterotetramers and are found in either form in several tissues, including heart and brain. All Kir channels are characterized by a minimal transmembrane domain architecture, namely two-transmembrane helical domains (M1-M2) comprising each of the four pore forming subunits. Within the membrane, the M1 and M2 helices harbor in between them the top third of the lining of the permeation pathway, the selectivity filter (SF), while the M2 helix forms the bottom two thirds of the permeation pathway (Fig. 1A, C). Kir channels are special among other channels in that their permeation pathway is extended by the cytosolic portion of the protein. Along the permeation pathway, three constrictions are seen, two are in the transmembrane portion (selectivity filter: SF, and helix bundle crossing: HBC), while the third is in the cytosolic (G-loop) (Fig. 1C). Evidence has implicated all three of these constrictions to be serving as gates. The SF gate serves a dual function: to select K^+ over other ions and to control ion flow as a gate by a) allosterically sensing the behavior of extracellular and intracellular parts of the channel protein near the membrane, and b) by adequate ion occupancy that keeps the opening patent or inadequate occupancy that causes the opening to collapse. The interactions that these gates make to stabilize themselves in an open or closed conformation, the role of stimuli and modulators in stabilizing or destabilizing specific conformations and the allosteric communication among gates are all areas of active research.

The membrane signals

G proteins: Membrane-associated GTP-binding (G) proteins transduce the action of extracellular signals (e.g. hormones, neurotransmitters) to effector proteins (e.g. phospholipase C, adenylyl cyclase, ion channels) by coupling to heptahelical transmembrane receptors called GPCRs (G Protein Coupled Receptors) (Milligan and Kostenis, 2006). Membrane-associated G proteins are heterotrimeric, composed of Gα and Gβγ subunits (the Gβ and Gγ subunits are always associated with each other as a Gβγ dimer under physiological conditions). Lipid modifications on the Gα subunit (palmitoylation / myristoylation - p) and on the Gγ subunit (geranylgeranylation / prenylation -gg) anchor the heterotrimeric G protein to the plasma membrane (Fig. 2A). Figure 2B depicts the G protein activation cycle. Binding of a ligand to the GPCR induces a conformational change to the receptor [(2)=>(3)] that is transduced to the Gα subunit, such that its affinity for intracellular GTP is greatly increased over the already bound GDP and in a Mg^{2+}-dependent manner GDP is exchanged with GTP [(3)=>(4)]. In this regard the activated GPCR is acting as a guanine nucleotide exchange factor (GEF) to stimulate the exchange of nucleotides with the Gα subunit. The Gα subunit uses the binding energy of GTP to stabilize its switch regions (Fig. 2A, SI-SIII) to produce a conformation that favors its dissociation from Gβγ and association with effector proteins [(4)=>(5), Fig. 2B]. Similarly, the dissociated Gβγ can also interact with effectors. Thus, the dissociated G protein subunits are activated to signal to downstream effectors. The activation of the G protein subunits ends by hydrolysis of the GTP to GDP by the GTPase activity of the Gα subunit (either intrinsic GTPase or stimulated by specific interacting proteins – e.g. GTPase activating proteins or GAPs, such as RGS proteins) [(5)=>(6)], which dissipates the energy that stabilized the switch regions and favors dissociation of the Gα subunit from the effector and re-association with Gβγ. The heterotrimeric G protein can interact again with the GPCR [(6)=>(1)] and the activation cycle can proceed again. Even though G protein signaling has been intensely studied for several decades, many questions remain regarding how G protein subunit – effector protein interactions control the activity of effectors in a signaling-dependent manner.

Phosphoinositides: Phosphoinositides (PIPs) are phosphorylated forms of phosphatidyl inositol (PI). PI is comprised of two fatty acid chains (arachidonyl is 20-carbon long and unsaturated, while stearyl is 18-carbon long and saturated) linked by a glycerol (G) moiety to a water-soluble inositol headgroup (Fig. 2C). Specialized lipid kinases and

phosphatases add and remove respectively phosphates at specific positions of the inositol ring to give rise to seven phosphorylated species: three different isomers of monophosphorylated PIPs, three different isomers of diphosphorylated PIPs and one species of a triphosphorylated PIP (Fig. 2C). The most abundant PIP in the plasma membrane is $PI(4,5)P_2$ that comprises about ~1% of the total phospholipid pool. It has been well appreciated since the 1970s, that phospholipase C (PLC) activation results in hydrolysis of PIP_2 into inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Fig. 2C). These two ubiquitous hydrolysis products of PIP_2 serve as important intracellular signals: IP_3 causes release of calcium from endoplasmic reticulum stores, while DAG activates membrane or membrane associated proteins, such as protein kinase C (PKC). On the other hand, since the 1990s, it has been appreciated that the added phosphates to PI confer a negative charge to PIPs that allows them to engage in electrostatic interactions with basic regions of proteins. These interactions range from non-specific to highly specific. A number of membrane-associated proteins utilize interactions with PIPs to localize to the plasma membrane and control events such as vesicular fusion to the plasma membrane, endocytosis, actin cytoskeleton dynamics, actions of AKAPs (A-kinase anchoring proteins), intracellular Gα (small G) protein activity by recruiting to the membrane GEFs and GAPs (Logothetis et al., 2011). Ion channels and transporters have been studied extensively with regard to their interactions with PIPs and their functional implications, since the pioneering work of Hilgemann (Hilgemann and Ball, 1996). It appears that such interactions are critical for the proper function of these transmembrane proteins by mechanisms that seem to be well conserved across the ion channel superfamily (e.g. Logothetis et al., 2010; Logothetis et al., 2011). It appears that control of transmembrane protein function by PIPs is not limited to ion channels and transporters but extends to other transmembrane proteins, such as single-pass growth factor receptors, like the epidermal growth factor receptor (Michailidis et al., 2011; Logothetis et al., 2011). Although the effects of PIPs on membrane-associated and transmembrane proteins has been studied extensively in the past couple decades, many open questions remain regarding the details of how the lipid-protein interactions regulate protein activity.

Membrane-delimited signaling

Soejima and Noma (1984) first used the cell-attached mode of the patch-clamp technique to study G protein-sensitive K^+ channels in heart. In these experiments, they

pioneered exchange of the pipette solution during a recording. They first recorded a low basal level of activity from rabbit atrial cells in the absence of acetylcholine (ACh) in the pipette (i.e. in the extracellular environment of the membrane patch) (Fig. 3A). This basal level of activity was not altered by applying ACh in the bath, suggesting that the channel could not be activated by generation of soluble second messengers. Yet, when they perfused the pipette with ACh, thus directly applying it to the extracellular side of the membrane, channel activity was dramatically stimulated. One can conclude from these experiments that the activation of K^+ currents by ACh (K_{ACh}) was confined to the membrane patch isolated by the pipette in the cell-attached recording and it required that ACh was present directly on the extracellular side of the membrane, where the ion channel activity was being recorded. This classic experiment by Soejima and Noma has been utilized as a key experiment in differentiating membrane-delimited from soluble second messenger signaling.

A year later, two laboratories demonstrated that G proteins transduced the ACh signal to the K^+ channels: Hille's group showed that pertussis toxin (PTX), which ADP ribosylates the Gi/o proteins and functionally uncouples them from the GPCR (the muscarinic type 2 –M2- receptor in this case), prevented agonist-induced K_{ACh} activation (Pfaffinger et al., 1985). Breitwieser and Szabo (1985) showed that inclusion of a non-hydrolyzable analog of GTP (GTPγS) in the patch pipette activated constitutively K_{ACh} (GTPγS diffuses into the cell from the patch pipette and activates the G proteins and thus the channels, irreversibly). Two years later Logothetis and colleagues in the Clapham and Neer labs (1987), showed that perfusion of purified G$\beta\gamma$ subunits in the inside-out mode of the patch-clamp technique could activate K_{ACh}, independently of the endogenous G proteins in atrial cells. K_{ACh} was the first effector shown to be activated directly by G$\beta\gamma$ and a model was proposed suggesting that following G protein subunit activation, the G$\beta\gamma$ subunits stimulated K_{ACh}, independently of the Gα subunits. Contrary to this conclusion, Yatane and colleagues in the Brown and Birnbaumer labs (1987) drew the opposite conclusion, namely that the activated Gα, and not the G$\beta\gamma$, subunits were responsible for K_{ACh} activation. A controversy arose that took seven years to settle in favor of the G$\beta\gamma$ hypothesis (Reuveny et al., 1994). Although the ability of G$\beta\gamma$ to stimulate native or recombinant G protein-sensitive K^+ channels has not been questioned since, a yet poorly defined involvement of Gα subunits in channel activation has been suspected by data produced in a number of different laboratories. This issue

will be re-visited below (under the heading “G protein-dependent channel gating”). In 1995, Krapivinsky and colleagues in the Clapham lab (2005) reported that the recombinant channels comprising the atrial K_{ACh} were a heteromeric assembly of GIRK1 and GIRK4 subunits.

In 1998, two reports from different laboratories showed that stimulation of G protein-sensitive channels depended on PIP_2 (Huang et al., 1998; Sui et al., 1998). Not only G protein stimulation depended on intact levels of PIP_2 but activation by intracellular Na^+ or Mg^{2+} ions, which were shown to be G-protein independent ways of gating GIRK channels, also depended on PIP_2. Interestingly, unlike other Kir channels that also exhibited PIP_2 dependence for their activity, GIRK channels could not be activated by PIP_2 alone but required the co-presence of $G\beta\gamma$, Na^+, or Mg^{2+}. Zhang and colleagues from the Logothetis lab (1999) used a chimeric strategy between GIRK4 and a Kir2.1 (a channel that could be activated by PIP_2 alone) to show that specific amino acid differences between the two channels mostly accounted for their differences in affinity for PIP_2. As seen in Figure 4A and 4B a point mutation in GIRK4 to the corresponding residue found in IRK1 (or Kir2.1) (GIRK4-I229L) was sufficient to strengthen the affinity of the GIRK4 channel so that it could now be activated by PIP_2 directly, without requiring the co-presence of G protein subunits or ions. In fact, both the $G\beta\gamma$ subunits, as well as the Na^+ and Mg^{2+} ions were shown to increase the channel affinity for PIP_2, suggesting that they gated the channel, at least in part, by strengthening channel-PIP_2 interactions. Figure 4C shows a model that suggests that GIRK channel gating to the open state requires PIP_2 and at least one other intracellular gating molecule, such as $G\beta\gamma$, Na^+ or Mg^{2+}. Physiologically under conditions that do not cause PIP_2 hydrolysis, it is likely that there is enough PIP_2 in the membrane available to interact with ion channels and support their activity. How might activity be inhibited when PIP_2 is hydrolyzed? Since internal PIP_2 could modulate Kir currents in inside-out patches (e.g. Fig. 4B), and PIP_2 is found exclusively in the inner leaflet of the plasma membrane, the membrane-delimited mode of current regulation by PIP_2 is clear. The experiment in Figure 5 tested whether the Soejima and Noma experiment (Fig. 3A) that established membrane-delimited signaling for G-protein signaling could also be applied for PIP_2 signaling (Zhang et al., 2003). Unlike G protein signaling, we can see that in a cell-attached recording, PIP_2 hydrolysis triggered by ACh application in the bath solution did decrease basal GIRK4 activity in a reversible manner (the outward current spike upon ACh application indicates that ACh is indeed hydrolyzing PIP_2, as its hydrolysis product IP_3 is causing Ca^{2+} release

from internal stores and activation of a transient calcium-activated Cl^- current endogenous to *Xenopus* oocytes, where this experiment was performed). Thus PIP_2 hydrolysis outside the patch allows the higher concentration of PIP_2 within the patch to diffuse across the seal of the pipette with the membrane patch and inhibit the GIRK4 current. The inability of G protein signaling to diffuse across the seal in the same manner as PIP_2 may mean either that the lipid modifications of $G\beta\gamma$ subunits prevents it from crossing the pipette seal or the more likely possibility that the G protein signaling system operates as a macromolecular complex of the GPCR / G proteins / channel, and it is not possible for such a complex to diffuse through the seal between the pipette and the cell membrane.

Physiological significance of each membrane-delimited signaling mechanism

Stimulation of K_{ACh} by PTX sensitive G protein signaling leads to hyperpolarization of the membrane potential towards the equilibrium potential for K^+ ions (E_K), which under physiological K^+ ion concentrations is near -90 mV. By hyperpolarizing the membrane potential toward E_K, vagal nerve activity, releasing ACh to nodal pacemaker and atrial cells in the heart, causes a slowing of the heart rate (Trautwein et al., 1958) through M2 receptor stimulation and activation of K_{ACh} via the PTX-sensitive G proteins.

How does PIP_2 hydrolysis affect K_{ACh}? Kobrinsky and colleagues in the Logothetis lab (2000) showed that ACh in atrial cells activates Gq signaling through appropriate muscarinic receptors that cause PIP_2 hydrolysis. Inhibition of Gq signaling or manipulations to strengthen channel-PIP_2 interactions attenuated current desensitization that occurred shortly following the activation of the current by the PTX-sensitive G proteins. Keselman and colleagues, in the same lab (2007), measured the kinetics of PIP_2 hydrolysis via a fluorescence reporter and compared them to those obtained from G protein-sensitive currents. They labeled isolated PH domains from PLCδ1 with variants of the Green Fluorescence Protein (Cyan: CFP, or Yellow: YFP) and transfected HEK-293 cells with them. Under resting conditions, the labeled PH domains bind PIP_2 in the plasma membrane and by virtue of being concentrated in the two-dimensional plane of the membrane they come close (< 100 nM apart). Energy from excitation at ~430 nM is transferred to YFP when it is < 100 nM apart so that YFP emits a fluorescence signal at ~530 nM. The CFP emission is clearly distinguishable at ~475 nM. At resting conditions, the CFP and YFP-labeled PH domains come close so that fluorescence resonance energy transfer (FRET) can occur (Fig. 6A). Upon perfusion of ACh, PIP_2 hydrolysis (to

IP_3 and DAG via PLCβ1 activation) causes translocation of the labeled PH domains to the cytosol, causing them to move farther away from each other in the three-dimensional space of the cytosol and therefore to decrease the FRET signal. In fact the CFP emission increases as energy is no longer transferred to YFP, while YFP emission decreases. Figure 6B shows these changes in CFP and YFP emission signals and also plots the YFP/CFP ratio as a measure of FRET. Keselman and colleagues used this FRET approach to monitor PIP_2 hydrolysis and relate it to its effects on GIRK currents in recordings where both signals were obtained simultaneously. Figure 6C shows results from such recordings with whole-cell records on top and FRET signals at the bottom. When GIRK4 channels were transfected in HEK-293 cells with M2 receptor and the labeled CFP- and YFP-PH domains (black traces), there was no FRET change and the current showed minimal decline during the ACh perfusion. In contrast, when M1 and M2 receptors were co-transfected together with the channel and labeled PH domains (red traces), there was a clear FRET decrease that correlated closely with a decrease in the current. These results were consistent with the conclusions drawn from the previous study (Kobrinsky et al., 2000), where Gq signaling produced current desensitization by hydrolysis of PIP_2.

Figure 7 shows how the two membrane-delimited signaling mechanisms converge at the level of the channel to modulate GIRK (Kir3) activity. The Gi/o-coupled muscarinic receptors activate GIRK currents via a G$\beta\gamma$-mediated mechanism, while the Gq-coupled muscarinic receptors inhibit GIRK currents via a PIP_2 hydrolysis mechanism. In heart, the same extracellular message, ACh, serves to activate both receptors that due to the intracellular steps they trigger the result is to first activate the channel (via G$\beta\gamma$) but secondly to partially inhibit this activation (via PIP_2 hydrolysis) and time overall the magnitude of the effect.

In the cardiac K_{ACh} example we can see the physiological value of coupling the two different membrane-delimited signaling mechanisms: a signal is produced that slows heart rate in a timed manner to ensure protection of the heart from shutting down for too long before it harms itself. Neuronal calcium channels, like K_{ACh}, have been shown to be both G$\beta\gamma$- and PIP_2-sensitive. How is integration of these two membrane-delimited signaling mechanisms serving physiology in other cases in the heart, brain, endocrine system, etc., where they are operative (e.g. including control of K^+, Ca^{2+}, or other channels or transmembrane proteins)? This question remains open to address.

PIP_2-dependent channel gating

Since PIP_2 seems to be absolutely required for the activity of all Kir channels (and most ion channels in general), I will discuss PIP_2-dependent channel gating first, as this seems to highlight a conserved gating machinery utilized in this family of channels that other modulators (e.g. G proteins, and others – see below) could affect as well.

A number of crystallographic complexes of membrane-associated proteins with PIPs have revealed two major generalizations (Rosenhouse-Dantsker and Logothetis, 2007).

1) All PIP-binding sites contain: basic residues with at least one lysine and in most of the structures at least one arginine; at least one residue with an aromatic ring, mostly tyrosine or histidine but tryptophan or phenylalanine residues are also seen; small polar residues such as serine or asparagine are also encountered.

2) The affinity of PIP binding seems to be directly related to the number of specific contacts made between the protein and the PIP, such that specificity may be determined by the relative binding affinity of the various PIPs. Thus, low specificity may indicate a low binding affinity as well.

The specificity of Kir channels to different PIPs was examined by Rohacs and colleagues in the Logothetis lab (2003). Water-soluble synthetic forms of PIPs (with eight carbon-long acyl chains) were used for $PI(3,4)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ to test the relative effectiveness of each species of PIPs in activating each Kir family member. The results from this study classified Kir channels in four groups with group 1 being the most, and group 4 the least stereospecific (Fig. 8A-D). Representative members from each group were studied using the water-soluble diC8-$PI(4,5)P_2$ and dose-response curves were constructed that showed that affinity and specificity were directly related for the most part (see above, generalization #2, Fig. 8E). Nishida and colleagues in the MacKinnon lab (2007) obtained a high resolution 3-D structure of a GIRK1 chimera between most of the cytosolic domains of the mammalian GIRK1 channel and most of the transmembrane domains of a bacterial Kir channel. Although they were not able to record activity from this chimeric protein, Leal-Pinto and colleagues in the Logothetis and Ubarretxena labs (2010) managed to functionally reconstitute the chimeric protein in planar lipid bilayers by including PIP_2 in the internal side of the lipid bilayer. The EC_{50} for diC8-PIP_2 activation of the GIRK1 chimera was 17 μM. Molecular Dynamics (MD) simulation studies by Meng and colleagues in the Cui and Logothetis labs suggest that PIP_2 engages in salt bridge interactions with twelve basic GIRK1 residues (K49, R52,

R66, K79, R81, K183, K188, K189, R190, R219, R229, R313) (Fig. 8F) (Logothetis et al., 2011). Interactions of PIP_2 with W80 have been reported by another modeling study on the GIRK1 chimera (Stansfeld et al., 2009). The type of residues identified are consistent with those seen in membrane-associated co-crystal structures with PIPs (see above, generalization #1). Mutation of these residues and their effects on PIP_2 sensitivity has not been probed yet in GIRK channels. Mutation of the equivalent positioned basic residues to neutral ones in Kir2.1 showed that all but two residues decreased PIP_2 sensitivity (Lopes et al., 2002). Whether these two residues contribute to the differences in PIP_2 sensitivity of the two channels remains to be determined. The distribution of these residues is insightful: in the N-terminus (K49, R52), the slide helix (R66, K79, R81), the C-linker (K183, K188, K189, R190 – connecting the C-terminus and the inner transmembrane helix), the CD-loop (R219, R229), the end of the G-loop gate (R313). It is likely that these channel regions are intimately involved in the gating process. In a recent study, Rosenhouse-Dantsker and colleagues in the Levitan and Logothetis labs set out to identify the molecular determinants accounting for differences in cholesterol sensitivity between two Kir2 channels, Kir2.1 and Kir2.3 (Rosenhouse-Dantsker et al., 2011). As with the GIRK1 chimera modeling studies, they identified critical residues that accounted for differences in cholesterol sensitivity between the two channels residing in the N-terminus, the C-linker, the CD-loop and the G-loop gate. These residues formed a cytosolic belt that surrounds the pore of the channel close to its interface with the transmembrane domain. Interestingly, modeling studies showed that cholesterol docked at sites that did not involve directly any of the identified residues, suggesting that the effect of cholesterol is transmitted to these sites allosterically. This result further strengthens the notion that the identified regions, harboring PIP_2 interacting residues and controlling allosterically cholesterol effects, are part of the gating machinery in Kir channels.

The regulatory role of the CD-loop in channel gating is best illustrated by the actions of intracellular Na^+ ions. GIRK2 and GIRK4 channels contain the motif DLR(K/N)SH in their CD-loops that binds intracellular Na^+, which as I discussed earlier can stimulate GIRK channels activity independently of G proteins (Rosenhouse-Dantsker et al., 2008). Rosenhouse-Dantsker in the Logothetis lab showed that Na^+ is in part coordinated by the side chains of the Asp and His residues. GIRK1 contains N217 in the corresponding position of the critical Asp residue and thus it lacks Na^+ sensitivity. R219 is predicted to be interacting with PIP_2. These studies showed that in the absence

of Na^+, the Asp and Arg residues form a salt bridge that is broken in the presence of Na^+, as it engages the Asp residue to coordinate it. This presumably frees the Arg residue in the CD-loop to interact with PIP_2 and activate the channel.

Figure 9 depicts the cytosolic surface of Kir channels (GIRK1: Fig. 9A-C; homology model of Kir1.1: Fig. 9D) as it faces the inner leaflet of the membrane. The transmembrane domains of the channels are not shown for clarity. Channel sites that have been identified to affect the sensitivity of these Kir channels to Gβγ (Fig. 9A, red), Na^+ ions (Fig. 9B, blue), phosphorylation by protein kinase A (PKA) and PKC (Fig. 9C, orange), and protons (Fig. 9D, green) are shown (Logothetis et al., 2007b). PIP_2 interacting residues are shown in cyan. The proximity of modulatory sites to those of PIP_2 is tantalizing, strengthening the notion that they all converge onto a common gating mechanism to regulate channel activity. Functional evidence also supports this notion. Du and colleagues in the Logothetis and Zhang labs (2004) provided evidence in Kir channels that distinct modulators, such as Gq and PKC activation, Gβγ subunits, Mg^{2+}, and pH, all depend on PIP_2 sensitivity to exert their effects. Mutations causing either stronger or weaker interactions with PIP_2 changed significantly the modulatory action of these signals. Additional studies on the PKA and PKC modulation of GIRK channels corroborated these conclusions and suggested that strategic placement of negatively charged phosphate groups via phosphorylation can exert either stimulatory or inhibitory effects by altering the same gating mechanism utilized by PIP_2 (Lopes et al., 2007; Keselman et al., 2007). These results have suggested that diverse modulators of channel activity and PIP_2 share a common gating mechanism.

G protein-dependent channel gating

I now turn lastly to G protein signaling with the ultimate goal to ask the yet unanswered question: how do G protein – channel (protein-protein) interactions gate the channel to the open state?

He and colleagues in the Logothetis lab (1999, 2002) employed a chimeric strategy between the Gβγ-sensitive GIRK4 and the Gβγ-insensitive IRK1 (Kir2.1). They identified two interesting residues: GIRK1(L333) [and its corresponding GIRK4(L339)] and GIRK4(H64) [and its corresponding GIRK1(H57)] that distinguished different aspects of G protein-dependent channel gating. Figure 10B shows the effect of the GIRK1(L333E) mutation (the Leu found in GIRK1 was mutated to Glu found in IRK1) and contrasts it to the control GIRK1 channel (Fig. 10A). Control GIRK1 exhibited significant basal (or ACh-

independent) currents and a three-fold increase in ACh-induced currents mediated through the M2 receptor. Gβγ co-expression, along with GIRK1 channel and M2 receptor, gave a two-fold increase in basal currents at the expense of the ACh-induced currents. In contrast, co-expression of the βARK-PH, which binds Gβγ and serves as its scavenger not allowing it to signal, greatly decreased basal activity and abolished ACh-induced activity. Gαi1 co-expression on the other hand, caused a similar reduction of the basal GIRK1 current but allowed significant ACh-induced activity, albeit at much lower levels than without co-expression of Gαi1. The GIRK1(L333E) mutant abolished ACh-induced activation of the channel in each of the expreriments described in the control (compare Figs. $10B_1$ to $10A_1$, $10B_2$ to $10A_2$, and $10B_4$ to $10A_4$). Moreover, the mutant did not show stimulation beyond basal levels by co-expression of Gβγ (compare Figs. $10B_2$ to $10A_2$). Yet, the mutant showed similar decrease in currents as the control channel by both Gβγ scavengers, βARK-PH and Gαi1 (compare $10B_3$ to $10A_3$ and $10B_4$ to $10A_4$). These results suggested that basal and ACh-induced activation were both Gβγ-dependent and that the L333 mutation interfered specifically with the ACh-induced activation but not the basal activity. Figure 10C-E shows experimental results that indicate that the N-terminal mutant H64F (shown for GIRK4, abbreviated G4) did the opposite, namely it affected basal but not ACh-induced activity. These experiments had to be conducted in the background of the GIRK4(I229L) mutation that strengthens channel-PIP_2 interactions (see Fig. 4A, B), as the GIRK4(H64F) mutation [or the corresponding GIRK1(H57F) mutation] abolished channel activity. In the background of the GIRK4(I229L) mutation, the H64F mutation reduced significantly basal currents. In fact, co-expression of the βARK-PH with the GIRK4(I229L) control reduced basal currents by the same relative amount as the H64F mutation did. In contrast, the H64F remaining currents were no longer sensitive to βARK-PH, indicating that this mutation indeed removed all Gβγ-dependent basal currents. Interestingly, the H64F mutation did not affect the ACh-induced currents compared to the control. Therefore, the H64F mutation (and the corresponding H57F in GIRK1 – see He et al., 2002) abolished specifically the Gβγ-dependent basal but not ACh-induced currents. In addition, these data argue that intact basal currents are a requirement for agonist-induced currents but the basal currents need not be dependent on Gβγ [as with the GIRK4(I229L)]. Thus, the underlying mechanism by which Gβγ elicits basal activity seems to be independent from the mechanism by which it yields agonist-induced activity.

In order to identify the position of the Gβγ-controlled gate along the transmembrane portion of the permeation pathway, Jin and colleagues in the Logothetis lab (2002) employed proline scanning mutagenesis in the M2 helix of GIRK4. Prolines found in α helices are known to induce kinks and the investigators reasoned that only kinks induced at specific positions of the helix ought to open the gate. This was indeed the case and activating prolines were positioned upstream of F187, the narrowest point of the transmembrane permeation pathway at the helix bundle crossing (HBC, see Fig. 1C). Proline kinks that opened the HBC gate (e.g. GIRK4-S176P) rescued the effects of mutations that disabled Gβγ gating, such as the GIRK4(H64F). Moreover, co-expression of Gβγ or βARK-PH had no effect when co-expressed with activating proline mutants. These results suggested that prolines opened the same transmembrane gate that Gβγ opens, namely the HBC gate.

Mirshahi and colleagues in the Logothetis lab (2002) approached the question of identification of key residues of interaction with the channel from the Gβγ side by using a similar chimeric strategy as was used with the channels. Unlike Gβ1γ2 that stimulates GIRK channel activity, Gβ5γ2 causes channel inhibition. Figure 11B shows a top-down view of the Gβ seven-blade propeller structure (compared to the side view seen in Fig. 2A). Chimeras between Gβ1 and Gβ5 pointed to blades 2 and 3 as the regions harboring the determinants of their differential actions on channel activity. Mutagenesis identified a number of residue differences between the two proteins that when mutated in Gβ1 reduced significantly or abolished its ability to stimulate GIRK channel activity. Three of these residues were surface exposed. S67 and T128 are shown in red and S98 in blue (Fig. 11A). Comparing surface representations of Gβγ with and without Gα (shown in green), it became clear that the red residues did not interact with the Gα subunits, while S98 did interact. In Figure 11C the researchers investigated the effects of these three point Gβ1γ2 mutants in their ability to affect the agonist-induced deficient GIRK channels (GIRK1-L333E with GIRK4-L339E). Both red residue mutations, unlike the blue residue mutation, showed significant reductions in the basal GIRK currents, as Gβ5γ2 did. These results suggested that basal channel activity could reflect interactions with Gβγ involving sites that do not interact with Gα. By analogy, agonist-induced activation may involve Gβ residues that do interact with Gα and become available only when the agonist triggers dissociation of Gα from Gβγ and association of the unoccupied residues with the channel.

GIRK channels are activated specifically by PTX-sensitive G proteins. How is this specificity conferred? Rusinova and colleagues in the Logothetis lab (2007) aimed to address this question. As it seemed that many different Gβ and Gγ combinations are capable of activating GIRK channels they explored whether differences between Gαi1 and Gαq could determine the specificity of Gβγ they associated with, which in turn when dissociated from Gα could activate the channel. Using again a chimeric approach between Gαi1 and Gαq, they found that the helical domain of Gαi1 was largely responsible for conferring specificity to Gβγ. Moreover, consistent with findings in other laboratories, Gαi1 bound the C-terminus of GIRK channels with higher affinity than Gαq. In fact, the helical domain of Gαi1 conferred a Gαi-like binding affinity for the C-terminus of the channel to chimeric Gαq subunits that contained it. These results assigned a special role to Gαi subunits in Gβγ stimulation of GIRK currents and confirmed that Gαi binds the channel just like Gβγ does. If both G protein subunits bind the channel how are they precisely controlling basal and agonist-induced activity? Is the role of the Gαi subunits active (a needed partner for Gβγ stimulation of the channels) or is it only passive (e.g. inhibitory) in scavenging the Gβγ subunits once they hydrolyze their GTP?

Crystal structures of GIRK channels with the Gβγ subunits have been elusive, despite significant efforts by a number of laboratories. As mentioned earlier in the previous section, Leal-Pinto and colleagues in the Logothetis and Ubarretxena labs (2010) successfully reconstituted the GIRK1 chimera in planar lipid bilayers. Figure 12A shows the arrangement for the lipid bilayer experiment. A lipid bilayer of known composition is painted in a small hole separating two chambers, into the trans (or external side) and the cis (or internal side) sides of the bilayer. The GIRK1 chimera purified in mild detergent is added from the trans side and with time it incorporates into the bilayer. With PIP_2 present in the cis side, once the chimera incorporates single-channel activity is observed. Functional reconstitution of the GIRK1 chimera allowed the experiment shown in Figure 12B to be performed. Addition of Gαi1 purified subunits on the cis side inhibited channel activity. Stoichiometric additions of Gβγ showed no change in activity but subsequent addition of GTPγS stimulated activity. In experiments where the GTPγS was added before Gβγ, it did not show an effect but addition of Gβγ, following exposure of the channel to activated Gα subunits, stimulated activity. These results suggested that both G protein subunits needed to be present and activated (Gα-GTPγS and Gβγ) for the GIRK1 chimera to become stimulated (Fig. 12C), suggesting that

activated Gα subunits have an active role in supporting Gβγ stimulation of channel activity. Additional work will be needed to examine whether purified mammalian GIRK channels (with no bacterial parts) behave similarly to the GIRK1 chimera. Additionally, could purified GPCR reconstitution establish signaling in this pure system and if so could the signaling complex behave in a manner comparable to cellular plasma membranes?

As can be appreciated by these studies, although significant progress has been made in the past 25 years that membrane delimited-signaling mechanisms have been described, fundamental questions about how protein-protein and lipid-protein interactions lead to channel gating remain unresolved. New tools and advances in approaching the problem using electrophysiology in plasma membranes and planar lipid bilayers, biochemistry, molecular biology, cell biology, structural biology, and computational chemistry have equipped us well to seek answers to the many remaining and long-standing questions.

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