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Review

Activation of inwardly rectifying potassium (Kir) channels by phosphatidylinositol-4,5-bisphosphate (PIP_2): Interaction with other regulatory ligands

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Abstract

All members of the inwardly rectifying potassium channels (Kir1-7) are regulated by the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP_2). Some are also modulated by other regulatory factors or ligands such as ATP and G-proteins, which give them their common names, such as the ATP sensitive potassium (K_{ATP}) channel and the G-protein gated potassium channel. Other more non-specific regulators include polyamines, kinases, pH and Na^+ ions. Recent studies have demonstrated that PIP_2 acts cooperatively with other regulatory factors to modulate Kir channels. Here we review how PIP_2 and co-factors modulate channel activities in each subfamily of the Kir channels.

Keywords: Kir channels; PIP_2; Regulation; Co-factors

Contents

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1. Introduction

A decade ago, it was reported for the first time that phosphatidylinositol-4,5-bisphosphate (PI-4,5-P_2 or PIP_2) serves as a second messenger that directly regulates Na^+–Ca^{2+} exchange and ATP-sensitive K^+ (K_{ATP}) channel activity in the cell membrane (Fan and Makielski, 1997; Hilgemann and Ball, 1996). Later on, many ion channels and transporters, such as inwardly rectifying K^+ (Kir) channels, voltage-gated K^+ (Kv) channels, voltage-gated Ca^{2+} channels (P/Q and N type), transient receptor potential (TRP) channels, two-pore-domain K^+ (K_{2P}) channels, and the cystic fibrosis transmembrane regulator (CFTR) have been found to be the targets of PIP_2 regulation (see reviews Hilgemann et al., 2001; Suh and Hille, 2005; Takano and Kuratomi, 2003). PIP_2 regulation has been most intensively investigated in the Kir channels. The direct interaction between the negative phosphate head-groups of PIP_2 and positively charged residues in N- and C- termini is essential for activation of channels (Fan and Makielski, 1997; Huang et al., 1998; Lopes et al., 2002; Schulze et al., 2003a; Shyng et al., 2000b). The channel–PIP_2 interaction may serve as the end effector regulating Kir channel activity. However, it is becoming clear that a variety of signaling partners influence Kir channel activity by modulating the interaction of Kir channels with PIP_2. For example, protein kinase A (PKA) phosphorylation enhances the interaction between Kir1.1 (ROMK1) and PIP_2. (Liou et al., 1999; Zeng et al., 2003). Similarly, G protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) and Na^+ stabilize the Kir3.1/4 (GIRK1/4)–PIP_2 interaction (Ho and Murrell-Lagnado, 1999; Huang et al., 1998; Zhang et al., 1999). Recently, we found that polyamines, which physiologically cause strong inward rectification in Kir2 channels, also act as co-factors in PIP_2 regulation of these channels. The PIP_2 interaction of the Kir6.2 (K_{ATP}) channel is regulated differentially by SUR subtypes (Song and Ashcroft, 2001). Conversely, PIP_2 also regulates the effects of channel ligands, such as ATP sensitivity in the K_{ATP} channels (Baukrowitz et al., 1998; Shyng and Nichols, 1998). It also affects the pH sensitivity of Kir1, Kir2, Kir4 and Kir6 channels. Here we review current understanding of the interaction between PIP_2 activation and other regulatory ligands in the subfamilies of Kir channels.

2. Regulation of channel activities by PIP_2

2.1. Structure of Kir channels

Kir channels include seven subfamilies (Kir1-7). The Kir1.x (ROMK) encodes the low conductance secretory K^+ channel in kidney (Hebert, 1995). The Kir2.x subfamily is the molecular counterpart of the I_{K1} current in cardiac myocytes (Dhamoon et al., 2004; Schram et al., 2003), which regulates resting membrane potential. The acetylcholine-activated K^+ channel (K_{ACh}) is a heterotetramer comprised of Kir3.1 and Kir3.4 (Krapivinsky et al., 1995) which regulates cardiac atrial electrophysiology. Heterotetrameric Kir4.1/Kir5.1 channels are expressed predominantly in the brainstem. Kir6.x together with sulfonylurea receptor (SUR) form the native ATP-sensitive potassium (K_{ATP}) channels (Inagaki et al., 1995), which are found in cardiac myocytes, pancreatic β-cells, skeleton and smooth muscle myocytes and neurons. Kir7 channels are widely expressed in brain, kidney, and intestine and provide a steady background K^+ current that regulates membrane potential (Krapivinsky et al., 1998b).

All Kir channels share a common structure consisting of intracellular amino (N) and carboxyl (C) termini and two putative membrane spanning segments (M1 and M2) flanking a pore-forming P-loop and signature sequence (*S*) (Ho et al., 1993; Kubo et al., 1993a) (Fig. 1A and B). The crystal structures of several K channels

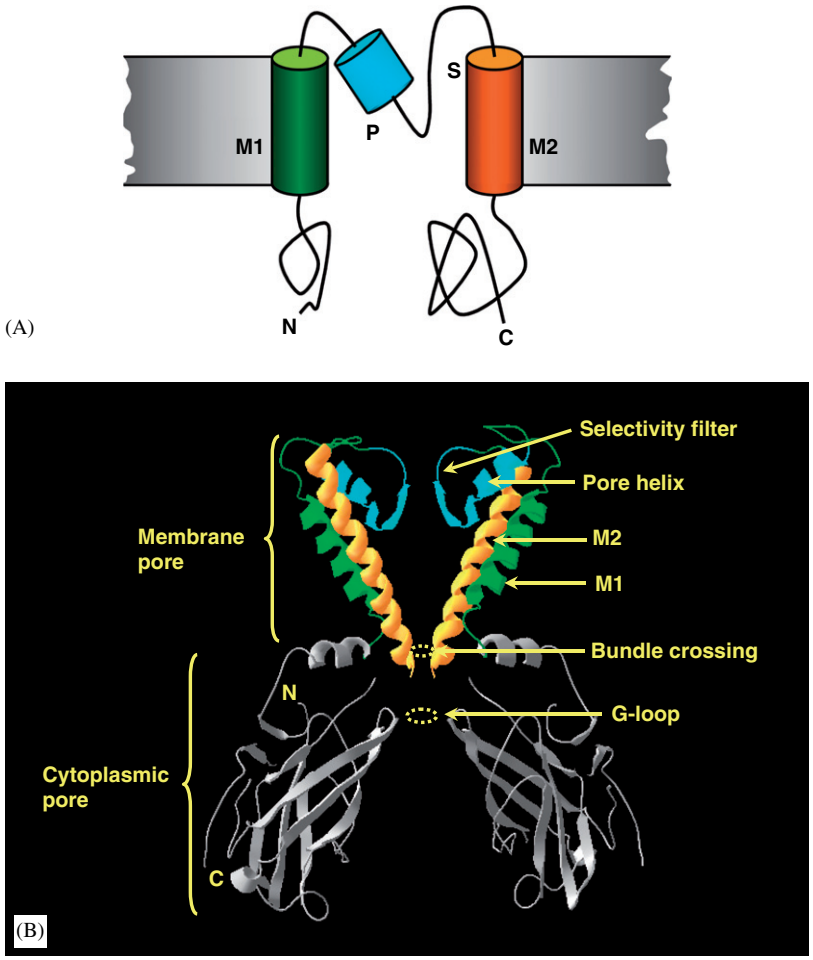

Fig. 1. Structure of Kir channels. (A). Topology of a Kir subunit. All the Kir channels share a common structure consisting of intracellular amino (N) and carboxyl (C) termini and two putative membrane spanning segments (M1 and M2) flanking a pore-forming P-loop and signature sequence (S). (B) The crystal structure of the bacterial KirBac1.1 channel showing the transmembrane domains of two subunits and the C-terminal domains of the other two subunits.

have been determined recently (Doyle et al., 1998; Kuo et al., 2003; Nishida and MacKinnon, 2002). It has been suggested that the Kir channel has two different "pores": a "transmembrane pore" formed by the P loop and M2 segment (~32 A long); and a "cytoplasmic pore" formed by the cytoplasmic N and C termini (~30 A long). The cytoplasmic pore region forms the binding site for the ligands and other regulators, thereby controlling access to the transmembrane pore. The M2 segments from four subunits cross near the cytoplasmic end of the transmembrane pore to form a "bundle crossing". The cytoplasmic pore contains a narrow G-loop (girdle) structure near the junction to bundle crossing (Pegan et al., 2005). The bundle crossing and G-loop may contribute to "lower gating" in Kir channels, while the selectivity filter controls "upper gating".

2.2. Activation of Kir channels by PIP_2

Activation by membrane phosphoinositides such as PIP_2 is a common feature of all Kir channels. The direct interaction between negative phosphate head-groups of PIP_2 and positively charged residues in N- and C-termini are essential for activation of channels (Fan and Makielski, 1997; Lopes et al., 2002; Schulze et al., 2003a; Shyng et al., 2000b).

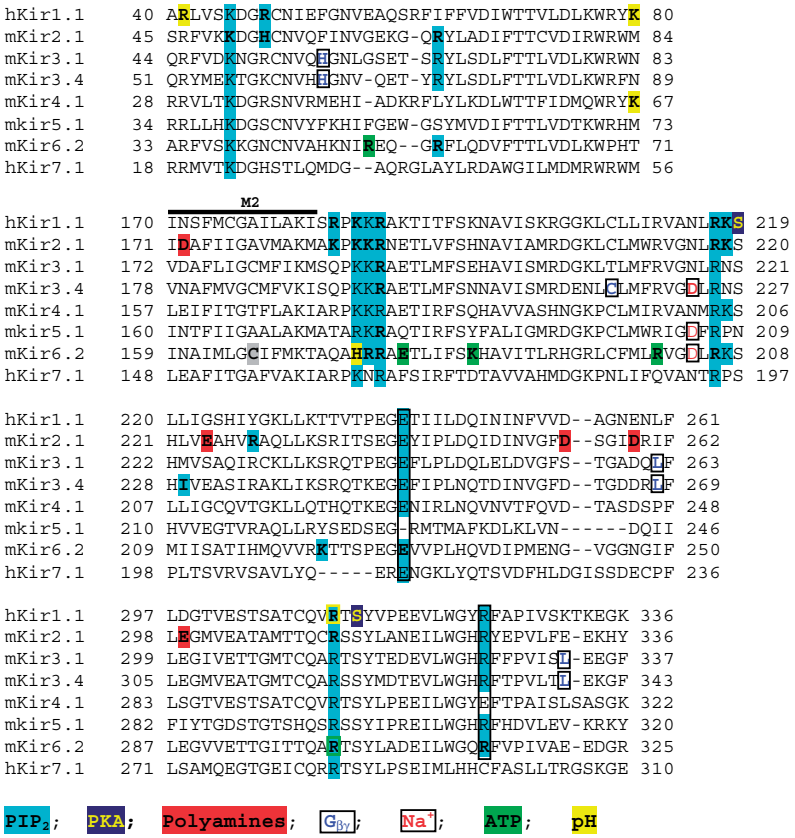

Fig. 2. Amino acid alignments of Kir1-7 channels. The Kir 1.1 and Kir7.1 sequences are from human (h) and others are from mouse (m). Parts of N-termini (the first segment), M2 helix (indicated by the horizontal bar above the second segment) and C-termini are shown. As indicated at the bottom, conserved residues critical for PIP_2 interaction are highlighted in cyan, with the identified ones shown in bold; PKA phosphorylation sites in the Kir1.1 are marked by yellow letters with black background; polyamine binding/interacting residues in Kir2.1 in red; residues critical for $G_{\beta\gamma}$ binding in Kir3.1 and Kir3.4 are shown as boxed blue letters; ATP binding residues in Kir6.2 channel in green; the pH-sensing residues are highlighted in yellow; See text for details.

Fig. 2 shows the amino acid alignment of representative members of the Kir1-7 subfamilies. The amino acid residues critical for the interaction with PIP_2 are highlighted in cyan. Whereas most are positively charged residues, located either in N terminus (e.g. R67, R82 in Kir2.1) or C-terminus (e.g. K187, K188, R189, R218, R312 in Kir2.1) (Fan and Makielski, 1997; Huang et al., 1998; Lopes et al., 2002; Schulze et al., 2003a; Shyng et al., 2000b), several uncharged residues may also play a role. For example, Zhang et al demonstrated that mutation of isoleucine at 229 in GIRK4 to the corresponding leucine in IRK1 (I229L) strengthened Kir3.4–PIP_2 interactions (Zhang et al., 1999).

Based upon their location in the cytoplasmic regions of the channel, the residues contributing to PIP_2 interaction can be divided into two groups. One group is located immediately beneath the inner leaflet of the plasma membrane and, presumably, interacts directly with PIP_2. In Kir6.2, this corresponds to residues R176, R177, and R206. The second group is further away from the membrane and probably affects PIP_2 interaction allosterically. An example in the Kir6.2 channel was reported by Lin et al. (2003), who suggested that E229/R314 ion pairs formed between adjacent subunits may stabilize the quaternary structure of the tetramer facilitating the ability of positively charged residues under the membrane to interact with membrane PIP_2. This inter-subunit interaction may also exist in other Kir channels since this ion pair is well conserved (boxed and highlighted in cyan in Fig. 2).

Each PIP_2 molecule consists of an inositol head group and fatty acid side chains. In contrast to naturally occurring PIP_2 in the cell membrane, short chain water soluble diC4 PIP_2 does not activate Kir channels,

suggesting the long fatty acid chains are required to activate Kir channels by incorporating PIP_2 into the membrane (Rohacs et al., 1999). The insertion rate of PIP_2 molecules into the membrane might explain the typically slow time course for their activating effects. It has also been reported that Kir3.1/Kir3.4 channels are activated more effectively by the natural arachidonyl stearyl PIP_2 than by the synthetic dipalmitoyl PIP_2, whereas Kir2.1 channels are activated equally by these two analogs. Moreover, the number and position of the phosphates in the inositol head group also affects the efficiency of PIP_2 activation of Kir channels. While K_{ATP} channels display low specificity to activation by different phosphoinositides (i.e. they can be equally activated by PI-3,4-P_2, PI-3,5-P_2, PI-4,5-P_2, and PI-3,4,5-P_3), all other Kir channels are activated preferentially by PI-4,5-P_2 over PI-3,4-P_2, and PI-3,4,5-P_3 (Rohacs et al., 2003). PI-4-P was much less effective than PI-4,5-P_2, whereas PI was inactive (Rohacs et al., 1999).

The putative location of the PIP_2 activation gate in the Kir channels has been studied by several groups. (Pegan et al., 2005; Phillips and Nichols, 2003; Proks et al., 2003; Xiao et al., 2003). Although the details are still controversial, it seems likely that PIP_2 binding to the cytoplasmic region (near the lower gate) of the channel may cause an overall conformational change in the channel pore to drive it to an open state. Possible sites for the critical conformational changes include the bundle crossing formed by the M2 helix (Xiao et al., 2003) and/or the G-loop (girdle) structure (Pegan et al., 2005), which may modulate the upper gate at the selectivity filter (Xiao et al., 2003).

3. Modification of PIP_2–channel binding affinity by other co-factors

Whereas PIP_2 may serve as the final controller of pore gating in Kir channels, accumulating evidence indicates that many co-factors contribute to the channel regulation by modulating the channel–PIP_2 interaction.

3.1. PKA augments PIP_2 effects in Kir1 channels

Protein kinase phosphorylation regulates the activities of various channel types, such as L-type Ca channels, delayed-rectifier K channels, cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, and Kir channels. Liou et al. (1999) found that PKA activates Kir1.1 channels in a PIP_2-dependent manner. In contrast to ATP, ATPγS can only serve as a substrate for PKA, but not as an activator of PI kinases. Therefore, PKA + ATPγS treatment can be used as a tool to enhance PKA phosphorylation without affecting PIP_2 levels in the membrane. It was shown that PIP_2, but not PKA + ATPγS, fully reactivated the channels after their rundown in membranes depleted of PIP_2, suggesting that PKA phosphorylation alone was not sufficient for channel activation. Direct application of PIP_2 liposomes activated Kir1.1 channels in a concentration-dependent manner, requiring 10 μM PIP_2 to fully reactivate the Kir1.1 channels following rundown. After pretreatment with PKA + ATPγS to phosphorylate the channels without PIP_2 regeneration, the concentration of PIP_2 required to activate the channels by ~90% was reduced, such that 1 μM PIP_2 fully reactivated the channels. Using anti-PIP_2 antibodies to inhibit channel activity by binding to PIP_2 in the membrane, thereby uncoupling its interaction with the channels, PKA + ATPγS pretreatment also attenuated the sensitivity of Kir1.1 channels to anti-PIP_2 antibodies. The observation that PKA phosphorylation lowered the concentration of PIP_2 necessary for activation of the channels suggests that the sensitivity of the channels to activation by PIP_2 is increased in phosphorylated channels. Further supporting this hypothesis, replacement of serine (S219, S313, shown as yellow letters with a black background in Fig. 2) with alanine at PKA phosphorylation sites (S219A, S313A) also reduced the channel's interaction with PIP_2. Phosphorylation of these two residues by PKA may be responsible for enhancing channel affinity for PIP_2. It was suggested that PKA phosphorylation might regulate channel function by inducing a conformational change (Levitan, 1994) or by adding negative charges to the protein molecule (Rich et al., 1993). However, the mutation of either Serine 219 or 313 to aspartate (S219D or S313D) did not substitute for the effect of PKA in enhancing PIP_2–channel interaction. Therefore, the enhancement of PIP_2–KIR1.1 interaction by PKA phosphorylation is most likely due to an allosteric effect (Fig. 3, Kir1.1 in the upper panel).

It has also been reported that PKC can regulate Kir1.1 channels in a PIP_2-dependent manner (Zeng et al., 2003). Unlike PKA, however, PKC inhibits channel activity. Phorbol myristate acetate (PMA), a PKC

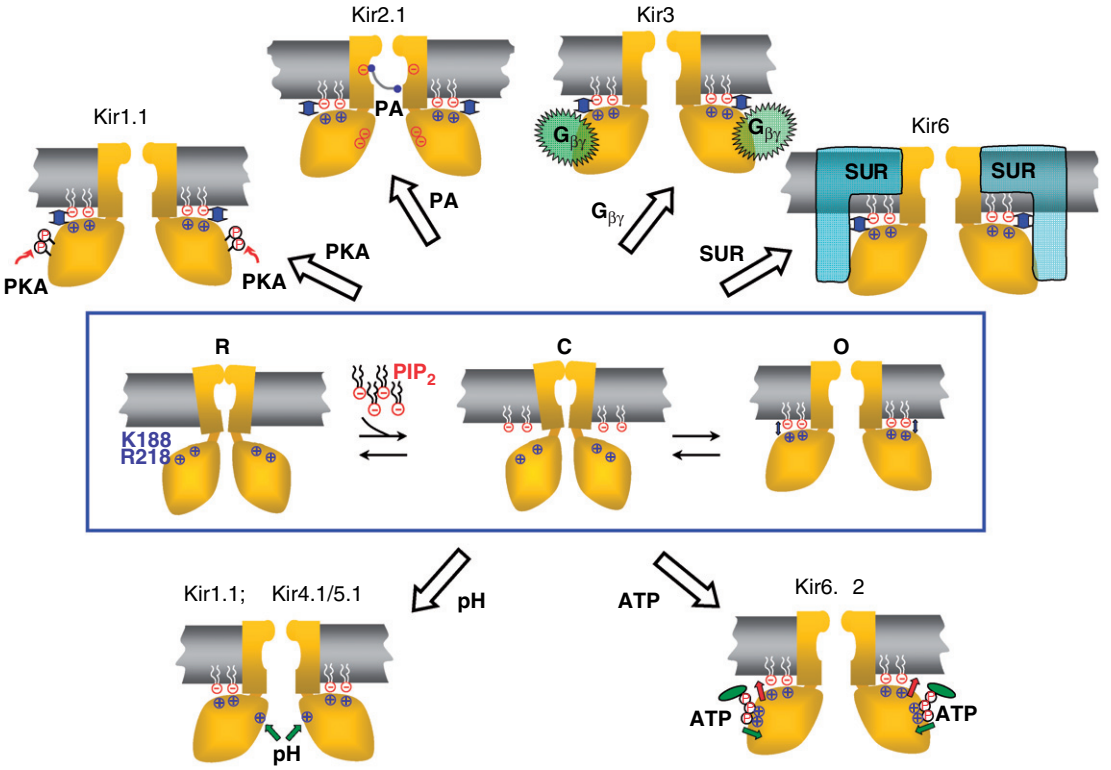

Fig. 3. PIP_2 activation and co-regulators of Kir channels. The scheme in the middle box shows the main transitions between open (O), close (C), and rundown (R) states in relation to the PIP_2-channel interaction in the Kir channels. The panels above demonstrate other regulators enhancing channel–PIP_2 interaction: in Kir1.1, PKA phosphorylation of S219 and S313; in Kir2.1, polyamines (PAs) interaction with D172, in Kir3, $G_{\beta\gamma}$ interaction with extensively the N- and C-termini; and in Kir6.2, SUR interaction with N terminus. The panels below demonstrate PIP_2 affecting ATP sensitivity in the Kir6.2 channel, and pH sensitivity in numerous Kir channels, respectively.

activator, was found to inhibit the activity of mutant Kir1.1 channels (S219A, R188Q). After inhibition by PMA in cell-attached patch recordings, application of PIP_2 liposomes to the cytoplasmic face of excised inside-out membranes restored channel activity. PIP_2 levels measured by ^{32}P labeling revealed that membrane PIP_2 content was reduced by PMA, and pretreatment with calphostin-C, a PKC inhibitor, prevented this reduction. Taken together, PKC appears to inhibit Kir1.1 channels by reducing membrane PIP_2 levels via an as yet unknown mechanism (Zeng et al., 2003).

3.2. *Polyamines strengthen Kir2.1 channel–PIP_2 interaction*

It was previously thought that the activity of the strong rectifier Kir2.1 channel depends solely on the presence of PIP_2 in the membrane without involving any other regulators (Hilgemann et al., 2001; Rohacs et al., 1999; Soom et al., 2001; Zhang et al., 1999). However, recently we found that polyamines, which cause inward rectification in Kir2 channels, also act as co-factors in PIP_2 regulation of channel activity (Xie et al., 2005).

Voltage-dependent block by intracellular polyamines and Mg^{2+} is the common mechanism underlying the inward rectification in all the Kir channels (Fakler et al., 1995; Ficker et al., 1994; Lopatin et al., 1994). Polyamine block has been most intensively studied in strong inward rectifier Kir2.1 channels, and exhibits a complex dependence on both voltage and polyamine concentration (Guo and Lu, 2003; Lopatin et al., 1995; Xie et al., 2002, 2003). Two different regions with negatively charged micro-environments are critical for binding polyamines and determining inward rectification characteristics (Fig. 2, highlighted in red): one is in the transmembrane pore involving D172 (Lu and MacKinnon, 1994; Stanfield et al., 1994; Wible et al., 1994;

Yang et al., 1995), and the other is in the cytoplasmic pore involving E224, E299, D255, and D259 (Fujiwara and Kudo, 2006; Kubo and Murata, 2001; Pegan et al., 2005; Yang et al., 1995). Several recent studies suggest a model in which positively charged polyamines can bind to the cytoplasmic region involving E224 and E299 without occluding the pore (Chang et al., 2003; Kubo and Murata, 2001; Xie et al., 2003). The pre-positioning of polyamines at the cytoplasmic pore then facilitates entry of polyamines into a deeper binding site located within the membrane pore at D172. Polyamines are most likely to occlude outward ion permeation at the selectivity filter (John et al., 2004; Kurata et al., 2004), although this point is still controversial (Shin and Lu, 2005).

In a recent study (Xie et al., 2005), it was shown that long polyamines also strengthen the interaction of PIP_2 with Kir2.1 channels, acting to maintain channel availability. Kir2.1 channels were overexpressed in *Xenopus* oocytes and macroscopic currents recorded in giant inside-out membrane patches. Channel activity ran down upon patch excision during superfusion of the patch with a Mg^{2+}- and polyamine-free solution. Channel activity could be recovered by applying exogenous PIP_2 (10 μM) after rundown. MgATP (2 mM) could also rescue the channel activity, probably by the production of PIP_2 via MgATP-sensitive PI kinases. Surprisingly, both spontaneous and PIP_2 antibody-induced rundown of Kir2.1 channels in excised inside-out patches was markedly slowed by long polyamines such as spermine and 1,10-decanediamine (DA10) or 1,12-dodecanediamine (DA12). In K188Q mutant channels, which have been shown to have a low PIP_2 affinity, application of PIP_2 (10 μM) was unable to activate channel activity in the absence of polyamines, but markedly activated channels in the presence of long diamines, while short diamines had no effect.

Neomycin is a polycation that binds PIP_2 (Arbuzova et al., 2000), preventing it from interacting with the channel. Therefore, neomycin sensitivity can be used to estimate PIP_2 binding affinity (Huang et al., 1998; Ribalet et al., 2005; Schulze et al., 2003a; Xie et al., 2005). Low neomycin sensitivity indicates strong channel–PIP_2 interaction, and conversely, high neomycin sensitivity reflects weak channel–PIP_2 interaction.

Using neomycin sensitivity as an indicator of PIP_2 affinity, we found that long polyamines strengthened the interaction of Kir2.1 wild type or K188Q channels with PIP_2. The negatively charged D172 residue inside the transmembrane pore region was critical for the shift of channel–PIP_2 binding affinity by long polyamines. We propose that in the absence of long polyamines, the channel opens (O state, Fig. 3) when the negative head charges of PIP_2 interact with positively charged residues in the cytoplasmic domain, such as K188, R218, etc., near the plasma membrane. It has been suggested that PIP_2 binding causes conformational changes at the bundle crossing formed by the M2 helix (Xiao et al., 2003) or the G-loop (girdle) near the junction between transmembrane pore and cytoplasmic pore domains (Pegan et al., 2005), which in turn promotes the open state. The channel closes when the interaction with PIP_2 is lost, either transiently (C state), or due to PIP_2 depletion (rundown, R state, boxed area in Fig. 3). When long polyamines are present, however, they interact with the channel in a manner dependent on the negative charges at D172 in the M2 region, but also involving other residues that remain to be identified. We speculate that this interaction stabilizes the bundle crossing in its open configuration, allosterically enhancing the interaction with PIP_2 at the cytoplasmic side of the membrane (Fig. 3, Kir2.1 in the upper panel). The bound PIP_2 molecule(s) are then protected from hydrolysis or binding to neomycin. This hypothetical schema is consistent with recent observations showing that PIP_2 binding to the cytoplasmic region links upper gating involving the M2 and selectivity filter regions (Xiao et al., 2003). We conclude that long polyamines are capable of stabilizing the channel in an open configuration, enabling an apparent increase of channel–PIP_2 binding affinity; thus, long polyamines serve a dual role as both blockers and co-activators (with PIP_2) of Kir2.1 channels (Xie et al., 2005).

Conversely, we also studied how the PIP_2–channel interaction affects polyamine block. Polyamine (DA10) blocking affinity and unblocking kinetics in the presence or absence of 100 μM neomycin (or 300 μg/ml poly lysine) were evaluated. Although the channel activity decreased by ~60% after neomycin application, neither DA10 blocking affinity nor unblocking time constant were altered (Xie et al, unpublished data). These results are consistent with the observation that the four subunits in the Kir2.1 channel function in a highly cooperative way.

3.3. $G_{\beta\gamma}$ and Na^+ stabilize interactions between PIP_2 and Kir3 channels

G-proteins can modulate ion channels indirectly via various second messenger signaling pathways or directly through membrane delimited pathways. An example of the latter is $G_{\beta\gamma}$ regulation of Kir3 channels,

a G-protein-regulated Kir subfamily consisting of five members (Kir3.1–3.5). Kir3.1 and Kir3.4 subunits were found to encode an heterotetrameric channel with similar electrophysiological properties and atrial distribution as the K_{ACh} channel (Dascal et al., 1993; Krapivinsky et al., 1995; Kubo et al., 1993b). The direct binding of $G_{\beta\gamma}$ to the N- and C-termini of Kir3.1–4 channel has been demonstrated using Kir3 fusion proteins. It is well accepted that a direct interaction between the N- and C-termini of Kir3 channels with $G_{\beta\gamma}$ is necessary for G-protein modulation of the channel (Doupnik et al., 1996; Finley et al., 2004; Huang et al., 1997, 1995; Inanobe et al., 1995; Ivanina et al., 2003; Krapivinsky et al., 1998a; Kubo and Iizuka, 1996; Kunkel and Peralta, 1995; Slesinger et al., 1995).

A novel finding in Kir3 channel regulation in the late 1990s was the PIP_2 requirement for the $G_{\beta\gamma}$ stimulation of the channel activity. Sui et al. (1998) showed that the $G_{\beta\gamma}$ activation of the Kir3.1/4 channels required the presence of PIP_2, suggesting a functional dependence of G protein signaling on membrane phospholipids. Direct application of $G_{\beta\gamma}$ to the cytoplasmic side dramatically slowed the inhibition of channel activity by PIP_2 antibodies, and augmented the activation of the channel by PIP_2. When Kir3.1/4 channels were allowed to run down completely, they were not activated by addition of $G_{\beta\gamma}$ alone. Application of PIP_2 reactivated them in minutes without $G_{\beta\gamma}$ and in seconds in the presence of $G_{\beta\gamma}$. Moreover, coexpression of $G_{\beta\gamma}$ with Kir3 channels slowed the inhibition of K^+ currents by PIP_2 antibodies by more than 10-fold. Thus, $G_{\beta\gamma}$ may promote a conformational change that strengthens channel–PIP_2 interaction and activates Kir3 channels. Mutant channels (R188Q) that interact weakly with PIP_2 did not open under control conditions, but could be activated when the interaction with PIP_2 was strengthened by adding $G_{\beta\gamma}$ subunits.

Where are the $G_{\beta\gamma}$ protein binding regions located in the Kir3 channels? So far, four separate regions have been identified (Finley et al., 2004; Ivanina et al., 2003), although the exact binding sites still remain to be determined. Region 1 contains part of the N-terminal domain. The entire C-terminal domain appears to contribute to $G_{\beta\gamma}$ binding: Region 2 is located at the proximal C-terminus and Region 3 at the middle C-terminus; Region 3 encompasses the ßL–ßM loop and surrounding amino acids, and has recently been demonstrated to be critical for $G_{\beta\gamma}$ binding (Finley et al., 2004); Region 4 may exist and is unique to the distal end of Kir3.1 channels. Single point mutation experiments have revealed several specific residues that are critical for $G_{\beta\gamma}$ binding and activation of Kir3 channels: such as H64 and L268 Kir3.4; H57 and L262 in Kir3.1 (He et al., 2002), L333 in Kir3.1, L339 in Kir3.4 (He et al., 1999), and C216 in Kir3.4 (Krapivinsky et al., 1998a) etc (Fig. 2, boxed blue letters). The 3-D crystal structure of the Kir3.1 cytoplasmic domain (Nishida and MacKinnon, 2002) makes it possible to estimate the geometry of the regions and/or single residues critical for $G_{\beta\gamma}$ binding. These studies have revealed that the main $G_{\beta\gamma}$ binding segments are exposed to the cytosol (outer edge of the cytoplasmic pore), in a position readily available to bind protein partners. Although no experimental evidence is available, the movements in the C terminus in response to $G_{\beta\gamma}$ binding could conceivably alter the structure of the PIP_2 binding region allosterically and affect its interaction with PIP_2 (Fig. 3, Kir3 in the upper panel).

Intracellular Na^+ ions also activate Kir3 channels (Ho and Murrell-Lagnado, 1999; Logothetis and Zhang, 1999; Petit-Jacques et al., 1999; Zhang et al., 1999). An aspartate residue within the proximal C-terminal region of Kir3.2 (D226) plays a crucial role in the Na^+-dependent activation of both Kir3.2 homomeric channels and Kir3.1–Kir3.2 heteromeric channels. When the aspartate residue was substituted for an asparagine, activation of mutant channels by Na^+ was lost. Kir3.4 also has an aspartate at the equivalent position (D223), whereas Kir3.1 has an asparagine (N217). Consistent with Na^+-dependent activation acting through this residue, Kir3.4, but not Kir3.1 channels, are sensitive to Na^+ activation. These aspartates are in close proximity to two positively charged arginines (R218, R228 in Kir2.1; R225, R235 in Kir3.4) that are important for the interaction of PIP_2 with the channel (Zhang et al., 1999). The most likely mechanism for Na^+ activation of Kir3.2 or Kir3.4 is that Na^+ directly interacts with D226 (in Kir3.2) or D223 (in Kir3.4) to reduce the negative electrostatic potential in the vicinity of the PIP_2 binding site and enhances the PIP_2–channel interaction. Similar activation effect by Na^+ was also suggested in the Kir6.2 channels (John et al., 2005), consistent with the conserved aspartate residue (D204) at the corresponding site of D223 in Kir3.4 (Fig. 2).

Sui et al. (1998) suggested two discrete gates located at the Kir3 channel pore, one of which is sensitive to PIP_2, and the other to $G_{\beta\gamma}/Na^+$. Both PIP_2 and $G_{\beta\gamma}$/Na are necessary to open both gates to make the channel permeable to K^+ ions. From the structural point of the view, however, the PIP_2-controlled gate is likely the

main gate determining channel opening. Therefore, molecules such as $G_{\beta\gamma}$ and Na^+ are most likely to function via an allosteric mechanism regulating the PIP_2's interaction with the channel.

3.4. SUR subtypes control sensitivity of K_{ATP} channels to phospholipids

K_{ATP} channel activities are inhibited by high intracellular ATP concentrations (and to a lesser extent ADP), thereby acting as sensors of the metabolic state of the cell (see a recent review article by Nichols, 2006). As metabolic sensors, K_{ATP} regulate excitability in a variety of cell types (Rodrigo and Standen, 2005), including insulin secretion in pancreatic beta cells (Ashcroft, 1988), vascular and smooth muscle tone (Brayden, 2002; Teramoto, 2006), skeletal muscle (Nielsen et al., 2003), cardiac (Noma, 1983) and neuronal excitability (Amoroso et al., 1990; Liss and Roeper, 2001).

K_{ATP} channels are heteromultimers composed of the pore-forming Kir subunit Kir6.1/Kir6.2 and the sulfonylurea receptor SUR1/SUR2 (Inagaki et al., 1995; Clement et al., 1997; Babenko et al., 1998). SUR is a member of the ATP-binding cassette (ABC) transporter family and has 17 transmembrane helices arranged in groups of 5, 6, and 6 called transmembrane domains (TMDs) 0, 1, and 2, respectively. SUR also has two nucleotide binding domains (NBDs), which are large intracellular loops responsible for nucleotide binding and hydrolysis. K_{ATP} channels are regulated by both ligands and pharmacological compounds such as nucleotides, sulfonylureas, and K^+ channel openers, which interact with various subunits and binding sites. ATP interacts with the Kir6.2 subunit and inhibits channel activity. MgADP/MgATP bind to NBDs of the SUR subunit and antagonize ATP inhibition, promoting activation of K_{ATP} channels at physiological and pathophysiological concentrations of ATP (Gribble et al., 1997; Shyng et al., 1997). Moreover, K_{ATP} channel stimulation by K^+ channel openers and block by sulphonylureas are both mediated by SUR subunits.

Both Kir6.2 and SUR subunits contain endoplasmic reticulum (ER) retention signals, and neither of subunit traffics efficiently to the plasma membrane when expressed alone. Co-expression of SUR together with Kir6.2 allows the ER retention signals to be masked by their partner subunit, permitting efficient insertion into the plasma membrane (Zerangue et al., 1999). By over-expressing the Kir6.2 subunit alone in mammalian cells, functional channels can be detected in low density, and it has been possible to characterize their biophysical and pharmacological properties (John et al., 1998). Similarly deletion of the last 26–36 residues containing the ER retention signal of the C-terminus of Kir6.2 (Kir6.2ΔC) enables its independent functional expression. When co-expressed with SUR, the open probability (Po) of the Kir6.2 channel increases from 0.1–0.2 to 0.3–0.5 and sensitivity to ATP is enhanced (John et al., 1998; Tucker et al., 1997). The N terminus and M1 region of Kir6.2 contribute to the assembly with SUR by interacting with TMD0 and the cytosolic loop between TMD0 and TMD1. This interaction modulates gating of the channel pore (Fig. 3, Kir6.2 in the upper panel) (Babenko, 2005; Babenko and Bryan, 2002; Chan et al., 2003; Schwappach et al., 2000).

Recently, it has been shown that SUR can increase the P_o of Kir6.2 via two mechanisms (Ribalet et al., 2006). One is a direct mechanism in which SUR enhances the interaction between R176/R177 and PIP_2. Biophysically, this results long-lasting bursts of openings. The second is an indirect mechanism, which is MgADP-dependent and may involve the distal N-terminus of Kir6.2. Moreover, different isoforms of SUR may confer different levels of PIP_2 interaction, since Kir6.2/SUR1 channels show lower sensitivity to PIP_3/PIP_2 than Kir6.2/SUR2A channels (Song and Ashcroft, 2001).

Taken together, these findings on the regulation Kir1.1 by PKA, Kir2.1 by polyamines, Kir3 by $G_{\beta\gamma}$, and Kir6.2 by SUR suggests a general mechanism by which various intracellular regulatory particles bind to or interact with the N- and/or C-termini of these channels to cause a conformational change which modulates gating. These conformational changes are likely to allosterically alter the structure or position of the PIP_2 binding region and affect the channels interaction with PIP_2.

This hypothesis is supported by a recent study examining PIP_2 interactions with mutant Kir6.2 channels. It was shown that the C166A mutation increases Po to near maximal by inducing constant bursting. In conjunction with mutations that modify the PIP_2 binding site, i.e. R176/R177, it was shown that the C166A gating effect required interaction of PIP_2 with R176/R177. Most likely, the mutation at C166 (Fig. 2, highlighted in gray) alters the orientation of the channel's cytoplasmic region in such a way to enhance the R176/R177–PIP_2 interaction, resulting in bursts of openings (Ribalet et al., 2006).

3.5. Effects of other negatively charged membrane lipids and derivatives

In addition to PIP_2, other negatively charged membrane lipids and derivatives also play roles in regulating the Kir channels. For example, K_{ATP} channels are activated by long-chain acyl–coenzyme A (LC-CoA) esters (e.g. oleoyl-CoA) (Fox et al., 2003; Liu et al., 2001; Rapedius et al., 2005; Rohacs et al., 2003; Schulze et al., 2003b) and phosphatidic acid (Fan et al., 2003). Unlike Kir6.2, however, other Kir channels (Kir1.1, Kir2.1, Kir3.4, and Kir7.1) have been found to be inhibited by LC-CoA. The inhibitory potency of the LC-CoA depends on the length of the side chains and the presence of the negative-charged phosphate on the CoA group. Biochemical studies show that PIP_2 and LC-CoA competitively bind to the C-terminal domains of Kir channels with similar affinity. Therefore the mechanism of LC-CoA inhibition of Kir channels, other than Kir6.2, has been attributed to their competitive displacement of PIP_2. These results suggest that changes in fatty acid metabolism, such as LC-CoA production, may affect cellular electrical activity. In fact, it has been found that the cellular levels of LC acyl-CoA increase during cardiac ischemia (van der Vusse et al., 1992), which may contribute to the activation of K_{ATP} channels during metabolic stress (Liu et al., 2001).

4. The strength of the channel–PIP_2 interaction controls channel regulation by other signaling or gating molecules

The aforementioned results provide examples showing how the Kir channel–PIP_2 interaction is modified by other co-factors. The interaction between PIP_2 and regulatory ligands can also be bidirectional, i.e. PIP_2 can regulate the effects of other regulatory ligands.

4.1. Modulation of pH sensitivity in multiple Kir subfamilies (Kir1.1, Kir2.3, Kir4.1, Kir6.2)

Although several Kir members (e.g.Kir1.1, Kir2.3, Kir4.1, Kir5.1, and Kir6.2) have been shown to be regulated by intercellular pH, Kir1.1 and Kir4.1 channels have much higher sensitivity to pH than the others. Intracellular acidification reversibly reduces the Po of Kir1.1 in the physiological range, thereby playing a key role in K^+ homeostasis during metabolic acidosis in the kidney (Giebisch, 1998; Hebert et al., 2005). In Kir1.1 channels, this pH gating has been attributed to a discontinuous RKR triad (R41, K80, and R311) in the N and C termini. K80 in the N terminus plays the major role in pH regulation, and is missing in pH-insensitive Kir channels. pH gating is thought to involve structural rearrangements in cytoplasmic domains and the P-loop of the Kir protein. Intracellular pH, through titration of K80, regulates opening of the channels through movements in both N and C termini (Schulte and Fakler, 2000; Schulte et al., 1998). The equivalent lysine residue (K67) is also critical for the pH sensitivity in the Kir4.1 channel. In other Kir members, different residues are critical for pH gating. In the Kir6.2 channel, H175 seems to be a genuine protonation site in Kir6.2 channels. A threonine residue in the N-terminus (T53) plays an important role in the Kir2.3 channel sensitivity to intracellular protons. Interactions between subunits may also be critical for pH sensitivity of Kir1 channels. A highly conserved R–E ion pair forming an intersubunit salt bridge was also found to modulate pH sensitivity (Leng et al., 2006).

Multiple studies demonstrate that PIP_2 plays an important role in the pH regulation of Kir channels (Du et al., 2004; Leung et al., 2000; Schulze et al., 2003a; Yang et al., 2000). Leung et al suggested that PIP_2 binding to Kir1.1 alters the pKa for pH gating. Disruption of Kir1.1–PIP_2 interaction in the R188Q mutant (equivalent to R176 in Kir6.2 discussed above) caused an alkaline shift in pH sensitivity. Disruption of PIP_2–channel interaction may either cause a direct effect on the structure or act via an allosteric mechanism, such as altering the local chemical environment around K80 which results in an alkaline shift of its effective pKa (Leung et al., 2000). The residues critical for pH sensitivity are highlighted in yellow in Fig. 2.

Accumulating evidence indicates that low affinity PIP_2 interaction is a prerequisite for pH sensitivity in some Kir channels. It has also been reported that for pH gating in the physiological range, the N-terminal PIP_2 interaction site must be absent. For example, introduction of an arginine into this site (I63R) increased PIP_2 binding affinity in the Kir1.1 channels and abolished the ability of the Kir1.1 channel to respond to physiological changes in intracellular pH (Schulze et al., 2003a). The PIP_2 dependence for pH inhibition was also observed in Kir2 (Du et al., 2004), heteromeric Kir4.1–Kir5.1 channels (PIP_2 decreased pH sensitivity of

the heteromeric Kir4.1–Kir5.1 by interacting with the Kir5.1 protein at R178) (Yang et al., 2000), and in Kir6.2 channels (R54 in N terminus act as an important region for PIP_2 interaction and pH gating) (Schulze et al., 2003a). Thus, channel–PIP_2 interactions may act like a switch that controls pH-induced inhibition of Kir channels (Fig. 3, left in the lower panel).

Despite causing a marked increase in activity, alkalinization to pH 9.4 did not alter the R188Q sensitivity to anti-PIP_2 antibodies, suggesting that the increase of Kir1.1 activity by alkalinization is not due to an enhanced PIP_2–channel interaction (Leung et al., 2000). Therefore, although both the regulation by PIP_2 and by pH induce conformational changes in the cytoplasmic domains of the channels, these two mechanisms appear to be separate. However, it is conceivable that the movements in the C terminus in response to changes in pH_i could alter the structure of the PIP_2 binding region and affect its interaction with PIP_2.

4.2. ATP and sulfonylurea sensitivity in the K_{ATP} channels

It is well known that PIP_2 dramatically decreases ATP sensitivity of K_{ATP} channels (Ashcroft, 1998; Baukrowitz et al., 1998; Ribalet et al., 2000; Shyng and Nichols, 1998; Xie et al., 1999). This control by PIP_2 may be important for the regulation of K_{ATP} channels by intracellular ATP under physiological condition. As for the underlying mechanism, some studies suggested that the phosphate groups of ATP and PIP_2 may directly compete for the same sites in Kir6.2 to cause an apparent reduction of ATP binding affinity (MacGregor et al., 2002; Shyng et al., 2000b). However, other data has shown that the interaction of ATP and PIP_2 with the intracellular domains involve different non-overlapping positive residues (Ribalet et al., 2003, 2005; Schulze et al., 2003a). A recent homology modeling and ligand docking study proposed that the phosphate tail of ATP interacts with R201 and K185 in the C-terminus of one subunit, and with R50 in the N-terminus of the adjacent subunit; the N6 atom of the adenine ring interacts with E179 and R301 in the same subunit (ATP-binding residues are highlighted in green in Fig. 2). In contrast, the PIP_2 binding sites in the Kir6.2 channel are located at R54 in the N-terminus and at R177, R178, and R206 in the C-terminus. Therefore, the direct competition for the same binding sites is unlikely to explain the effect of PIP_2 on lowering the sensitivity of the K_{ATP} channel to ATP. Alternatively, an allosteric model can be proposed as follows. The negatively charged head group of membrane PIP_2 interacts with positive charged residues in the cytoplasmic domain of the K_{ATP} channel. This interaction may distort the ATP-binding site and prevent ATP from binding (Fig. 3, right in the lower panel). When PIP_2 is absent, however, the channel is able to bind ATP easily and causes it to close. Consistent with this idea, mutation of PIP_2 interacting sites in Kir6.2 (e.g. R176A) attenuated the effects of PIP_2 on ATP inhibition.

Membrane PIP_2 levels may change in response to alterations in phospholipid metabolism: stimulation of PI-kinases increase PIP_2 levels, whereas PIPs-phosphatases and phospholipases (e.g. phospholipase C, PLC) break down PIP_2 and decrease PIP_2 content (Jenkinson et al., 1994; Willars et al., 1998). In K_{ATP} channels, it has been shown that activation of PLC increased ATP inhibition (Baukrowitz et al., 1998; Xie et al., 1999), whereas overexpression of a PI-kinase reduced ATP sensitivity (Shyng et al., 2000a). Taken together, these findings suggest that levels of membrane PIP_2 can significantly change in vivo in response to various signaling pathways. This dynamic alteration of PIP_2 levels and its contribution to the modification of channel function has been described in many other channels.

Application of PIP_2 attenuates inhibition of K_{ATP} channels by glibenclamide, suggesting PIP_2 affects the interaction of SUR with Kir6.2 (Koster et al., 1999; Krauter et al., 2001; Ribalet et al., 2000). It is still not clear whether this effect is due to a direct PIP_2 effect on SUR–Kir6.2 interaction site, or mediated by a indirect effect, such as the increase in Po induced by PIP_2. The latter is more likely since mutation of PIP_2 binding sites in Kir6.2 (R176A) subunit also attenuated glibenclamide inhibition (Krauter et al., 2001).

Thus PIP_2 modulates both glibenclamide inhibition in the presence of SUR, and ATP sensitivity of the Kir6.2 subunits. Both types of modulation may involve PIP_2's interaction with Kir6.2 subunits, because a mutation in Kir6.2 (R176A) attenuated simultaneously the effects of PIP_2 on ATP and glibenclamide inhibition.

4.3. Other modulators affecting membrane PIP_2 level (PLC-linked receptor stimulation, PKC, Mg^{2+})

The strength of channel–PIP_2 interactions also controls the sensitivity of Kir channels to regulation by several other modulators of channel activity, such as PLC-linked receptor stimulation, PKC, or intracellular

Mg^{2+}. For example, Kir2.3 channels are inhibited by PLC-linked receptor stimulation, PKC or intracellular Mg^{2+}, while Kir2.1 channels are unaffected. This is probably due to their different PIP_2 affinities: Kir2.1 has a stronger interaction with PIP_2 than Kir2.3. Mutations of Kir2.1 which lower PIP_2 affinity (e.g. R312Q) become similarly sensitive to these regulators (Du et al., 2004). PLC-linked receptor stimulation, PKC, or intracellular Mg^{2+} are all thought to cause reduction of membrane PIP_2 levels via hydrolysis of PIP_2 (Zeng et al., 2003). Since channels with low PIP_2 affinity are more sensitive to changes in PIP_2 levels. This may explain their susceptibility to modulation by these regulators.

Although the extent to which membrane PIP_2 content changes dynamically under physiological conditions still remains questionable in the heart (Nasuhoglu et al., 2002), it has been shown that Kir channels can be regulated by PLC-linked receptor stimulation via PIP_2 metabolism in both cultured cell lines (Xie et al., 1999) and cardiac myocytes (Cho et al., 2005, 2001; Haruna et al., 2002).

5. Summary

PIP_2 modulates the function of Kir channels by directly interacting with cytoplasmic domains of these channels. This interaction may induce allosteric changes in both of the channels pores: the cytoplamic pore formed by the cytoplasmic N- and C-termini and the membrane pore which includes the M2 transmembrane domain and selectivity filter. Other regulators such as channel phosphorylation, polyamines, $G_{\beta\gamma}$, pH and ATP may also allosterically alter the channel structure in a manner that affects the PIP_2 binding region. Therefore, as summarized in Fig. 3, the interactions between Kir channels, PIP_2 and other regulators can have concerted effects and play a pivotal role in the regulation of Kir channels under physiological and pathophysiological conditions. Given the extremely important roles played by phospholipids and their co-factors in regulating Kir channel activities, the physiology of these channels cannot be understood without considering their interactions with membrane lipids.

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