# How Highly Charged Anionic Lipids Bind and Regulate Ion Channels

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The modulation of channel activity by direct interaction with membrane lipids is now an emerging theme in ion channel biology. In particular, phosphoinositides such as phosphatidylinositol 4,5-bisphosphate ($PIP_2$) are known to regulate the activity of most major classes of ion channel, as well as a number of other membrane transport proteins. The regulation of inwardly rectifying (Kir) potassium channels by $PIP_2$ is one of the most intensively studied and best-defined model systems for studying channel–lipid interactions. In this review we explore the mechanisms that underlie the complex regulation of Kir channels by phosphoinositides and other highly charged anionic lipids such as long chain CoA esters. Intriguingly, these different lipids result in either activation or inhibition depending on the Kir channel subtype. We describe how recent studies have provided structural insight into these lipid-induced gating movements, and discuss how this gating mechanism is central to Kir channel physiology.

## Introduction

As pointed out in the preceding perspective by Lundbæk (see p. 421), many lipids and lipophilic compounds alter ion channel function by affecting the collective physical properties of the membrane, i.e., the regulation does not depend on specific interactions with the channel. However, the regulation of potassium channels by highly negatively charged lipids such as phosphoinositides and long chain acyl-CoA esters is mechanistically distinct because these lipids bind directly to the channel and any effects mediated by changes in the physical properties of the bilayer itself are considered to be minimal.

The direct interaction of highly charged anionic lipids with ion channels is now emerging as a fundamental mechanism of channel regulation. A considerable body of work over the past decade has shown that members of almost every class of ion channel can be regulated by phosphoinositides, in particular phosphatidylinositol-4,5 bisphosphate (hereafter referred to as $PIP_2$) (Hilgemann et al., 2001; Suh and Hille, 2005; Gamper and Shapiro, 2007a). However, the molecular mechanisms underlying this form of direct lipid regulation are best understood in the family of inwardly rectifying potassium (Kir) channels and so will form the focus of this perspective.

## Regulation of Kir Channels by Highly Charged Anionic Lipids

In 1996/1997 three papers were published that provided the first evidence that highly negatively charged anionic lipids can modulate the activity of Kir channels. Hilgemann and Ball (1996) demonstrated that phosphoinositides such as $PIP_2$ activate native cardiac $K_{ATP}$ channels. In addition, Fan and Makielski (1997) showed that several cloned Kir channels, (e.g., Kir1.1, Kir2.1, and $K_{ATP}$ [Kir6.2/SUR1] channels) are activated by $PIP_2$. Furthermore, Larsson et al. (1996) demonstrated activation of native $K_{ATP}$ channels by long chain acyl-CoA esters (LC-CoA), which are metabolites of fatty acid metabolism.

It is now clear that all Kir channels are regulated by phosphoinositides (Logothetis et al., 2007a) and long chain CoA esters (Rapedius et al., 2005), and that the structure of the highly negatively charged head group present in these lipids is central to their ability to affect Kir channel activity. The anionic head group is thought to interact directly with a distinct positively charged binding site on the channel (Logothetis et al., 2007a) and can induce quite rapid gating motions (Rapedius et al., 2007a). Thus, the anionic head group appears to gate Kir channels very much like a classical ligand, with the only distinction that the ligand is tethered to the membrane. Interestingly, the functional consequences of these head group interactions are markedly different between members of the Kir channel family. For example, whereas $PIP_2$ activates all members of the Kir channels, LC-CoA esters inhibit their activity, except for $K_{ATP}$ channels, which are activated by LC-CoA (Rapedius et al., 2005).

The main issues that will be discussed in this perspective center around the following questions. (a) Where and how do anionic lipids interact with Kir channels? (b) What is the mechanistic basis for the different effects of $PIP_2$ and LC-CoA on Kir channels? (c) What are the conformational changes induced by the binding of these lipids? (d) What is the physiological relevance of this regulation?

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Abbreviation used in this paper: $PIP_2$, phosphatidylinositol 4,5-bisphosphate.


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### A Variety of Highly Charged Anionic Lipids Activate $K_{ATP}$ Channels

The effects of various lipids on cloned Kir6.2/SUR1 ($K_{ATP}$) channels have been evaluated by their application to the cytoplasmic membrane side in excised patches. From these studies it became clear that neutral lipids (e.g., phosphatidylethanolamine, phosphatidylcholine) or weakly negatively charged lipids (e.g., phosphatidylinositol) have little effect on channel activity (Fan and Makielski, 1997; Krauter et al., 2001), whereas lipids with highly negatively charged headgroups strongly activated $K_{ATP}$ channels. These were the different phosphoinositides including PI(4)P, PI(4,5)$P_2$, PI(3,4)P2, and PI(3,4,5)$P_3$, which all restored channel activity when applied after rundown, a process that typically occurs in excised patches due to the breakdown of endogenous phosphoinositides (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Baukrowitz et al., 1998; Shyng and Nichols, 1998; Rohacs et al., 2003). However, it is $PIP_2$ that is most important as it represents the major phosphoinositide in eukaryotic membranes comprising ~1% of the total phospholipids and is >100-fold more abundant than the other phosphoinositides such as PI(3,4)$P_2$ and PIP(3,4,5)$P_3$ (Gamper and Shapiro, 2007a).

LC-CoA esters (e.g., oleoyl-CoA, palmityl-CoA) also potently activate $K_{ATP}$ channels at low concentration (<1 μM) (Larsson et al., 1996; Gribble et al., 1998; Liu et al., 2001; Schulze et al., 2003b; Branstrom et al., 2004) (Fig. 1 C). Here the negative charge is contained in the CoA headgroup (structure see Fig. 1) and the 3′-ribose phosphate is particularly important as its removal abolishes any stimulatory effect on $K_{ATP}$ channels (Rapedius et al., 2005). Furthermore, a negatively charged artificial lipid (DOGS-NTA) also strongly activates $K_{ATP}$ channels (Krauter et al., 2001), indicating that the specific structure of the charged head group is not critical. However, other Kir channels exhibit much greater specificity (see below).

Screening of the negative charge in these lipids by divalent cations (e.g., $Mg^{2+}$) or their complexation by polycations such as polylysine or neomycin abolishes their stimulatory effect, suggesting that electrostatic interactions between the negatively charged headgroup and the channel are critical (Fan and Makielski, 1997; Schulze et al., 2003a,b). However, application of the head group itself (i.e., $IP_3$ or CoA) does not activate $K_{ATP}$ channels (Shyng and Nichols, 1998; Schulze et al., 2003b), demonstrating that lipid tethering of the charged head group to the membrane surface is required.

### A Conserved Binding Site for Highly Charged Anionic Lipids in Kir Channels

Systematic mutagenesis of basic residues in the cytoplasmic domains of Kir channels has identified several potential $PIP_2$-interacting residues that are highly conserved among the different Kir channels (Logothetis et al., 2007a). Neutralization of these residues results in channels with a low open probability that increases upon application of exogenous $PIP_2$. Furthermore, these mutant channels show increased sensitivity to inhibition by polylysine and neomycin, as well as faster inhibition by $PIP_2$-specific antibodies, suggesting that these mutations reduce the apparent $PIP_2$ affinity (Huang et al., 1998; Lopes et al., 2002; Schulze et al., 2003a). When mapped onto structural models of Kir channels these basic residues cluster in a region close to the membrane interface suggestive of a binding site (for details see Haider et al., 2007; Logothetis et al., 2007a). Each of the four subunits of these tetrameric channels likely contains one binding site. A recent study on Kir2.1 channels suggest that binding of $PIP_2$ to one of the four sites is sufficient to promote channel opening, and $PIP_2$ interaction with the remaining sites further increases the open probability in a cooperative fashion (Xie et al., 2008).

The structure of the $PIP_2$ binding site is therefore beginning to emerge. However, extensive mutagenesis studies have shown that a number of other residues located at positions quite distant from this site can affect the apparent $PIP_2$ affinity (Logothetis et al., 2007a). Some of these residues are uncharged and either alter the structure of the $PIP_2$ binding site allosterically, or interfere with the transduction mechanism that couples $PIP_2$ binding and channel opening. The latter effect would also change the apparent $PIP_2$ affinity as measured with macroscopic currents that are unable to distinguish between changes in ligand affinity versus changes in ligand efficacy.

Interestingly, mutation of the conserved $PIP_2$-interacting residues within the $PIP_2$ binding site also reduce the apparent affinity for LC-CoA in $K_{ATP}$ channels (Fox et al., 2003; Schulze et al., 2003b). In agreement, biochemical experiments on the C-terminal domains of Kir channels show that LC-CoA can competitively antagonize the binding of $PIP_2$ (Rapedius et al., 2005). In addition, the functional effects of $PIP_2$ and LC-CoA on $K_{ATP}$ channels are similar since both lipids strongly antagonize ATP inhibition, as well as inhibition by sulphonylureas (e.g., glibenclamide) and activation by channel openers (e.g., diazoxide) (Schulze et al., 2003b). These pharmacological compounds affect $K_{ATP}$ (Kir6.2/SUR) channels via interaction with the SUR subunit. These results suggest that $PIP_2$ and LC-CoA probably regulate $K_{ATP}$ activation via the same mechanism and the same interaction site.

### Bidirectional Regulation of Kir Channels by Phosphoinositides and LC-CoA Esters

As stated above, all tested highly charged anionic lipids activate $K_{ATP}$ channels with similar potency. However, the $K_{ATP}$ channel is the exception, as all other Kir channels show a bidirectional regulation by these different lipids, i.e., while $PIP_2$ activates all Kir channel subtypes, LC-CoA

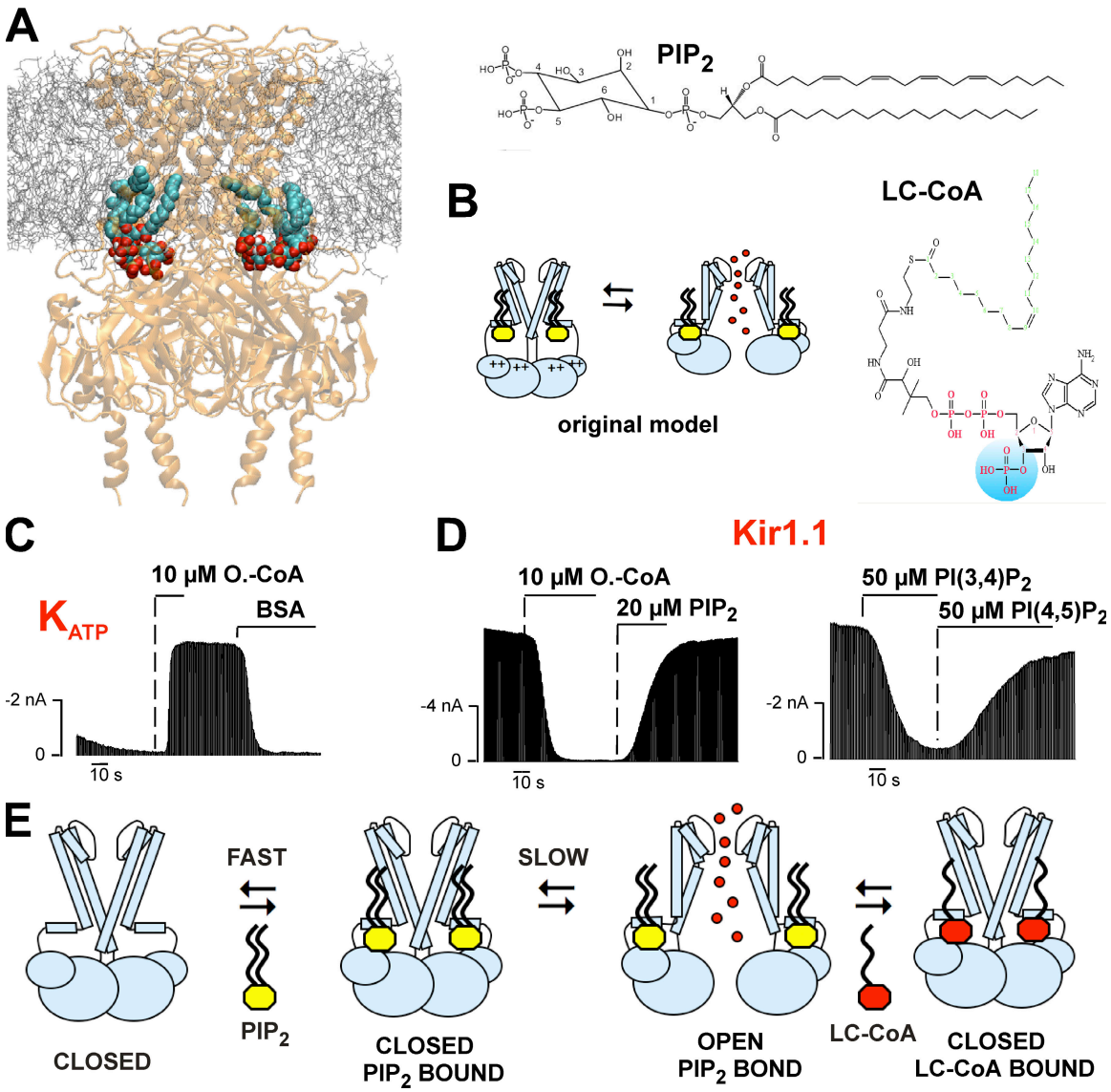


**Figure 1.** Proposed mechanism of phosphoinositide and LC-CoA gating in Kir channels. (A) A homology model of Kir6.2 in the closed state showing the location of the four bound $PIP_2$ molecules at the membrane/cytoplasm interface (position of $PIP_2$ was determined by a computational docking procedure (Haider et al., 2007). The negative charges on the headgroups (oxygen atoms in red) interact with a positively charged cluster in the cytoplasmic domains, for clarity the multiple residues that comprise the binding site are not shown, for details see Haider et al. (2007). (B) Previous models suggested that opening of the Kir channel is produced by electrostatic attraction between $PIP_2$ and its binding site that induces a major movement of the cytoplasmic domain toward the membrane. (C) In addition to being activated by $PIP_2$, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ (not depicted), $K_{ATP}$ channels are activated by oleoyl-CoA, a long-chain acyl-CoA ester as measured in inside-out patches from *Xenopus* oocytes. BSA is used to rapidly remove oleoyl-CoA. (D) Kir1.1 is activated by $PI(4,5)P_2$ but inhibited by oleoyl-CoA and $PI(3,4)P_2$ and inhibition could be reversed by $PIP_2$. (E) In contrast to the original model (B) we propose that all phosphoinositides and LC-CoAs can bind to the $PIP_2$ binding site on the closed Kir subunit. However, only certain bound lipids (e.g., $PIP_2$) are subsequently able to initiate the allosteric changes required for channel opening. Those that cannot induce channel opening (LC-CoA and $PI(3,4)P_2$) inhibit channel activity by competitive displacement of $PIP_2$. The chemical structures of $PIP_2$ and LC-CoA are shown at the top of the figure. The critical phosphate on LC-CoA required for its ability to induce opening in $K_{ATP}$ channels and inhibition in, e.g., Kir1.1 channels is highlighted in blue. Panel A is adapted from the results of Haider et al. (2007). C and D are adapted from Rapedius et al. (2005).

as well as some phosphoinositides have the opposite effect and inhibit Kir channel activity (Rohacs et al., 1999, 2003; Rapedius et al., 2005; Shumilina et al., 2006). To determine the apparent affinity (i.e., potency to activate) of a particular phosphoinositide Logothetis and coworkers established an assay using short chain fatty acids phosphoinositides such diC8(octanoly)-$PIP_2$ (Rohacs et al., 1999, 2003). These short chain phosphoinositides are soluble in aqueous solution as monomers and rapidly establish a water–membrane partitioning equilibrium allowing different diC8-phosphoionositides to be compared at a similar membrane concentration.

This assay revealed that $PIP_2$, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ activate $K_{ATP}$ channels with a similar potency, whereas all other Kir channels exhibit varying degrees of phosphoinositide specificity. For example, $PI(3,4,5)P_3$ produces little activation, and $PI(3,4)P_2$ no activation, of Kir1.1 and Kir2.1 channels when applied to closed channels (i.e., after rundown) (Rohacs et al., 1999, 2003). Intriguingly, $PI(3,4)P_2$ when applied to active channels (i.e., before rundown) produced marked inhibition of Kir1.1 and Kir2.1 that could be reversed by $PI(4,5)P_2$ (Rapedius et al., 2005) (Fig. 1 D). Even more strikingly, LC-CoA esters such as oleoyl-CoA potently inhibit all Kir channels (except $K_{ATP}$ channels) in a way that can be reversed by $PIP_2$ (Rapedius et al., 2005) (Fig. 1 D). Furthermore, removal of the 3′-ribose phosphate in LC-CoA that prevented activation in $K_{ATP}$ channels also abolished inhibition in Kir1.1 and Kir2.1 (Rapedius et al., 2005), indicating that this phosphate group is critical for Kir channel interaction.

### How Can the Same Lipids Act as Both Activators and Inhibitors of Different Kir Channels?

As described above, LC-CoA appears to bind to the same conserved phosphoinositide binding site that is found in all Kir channels. In a previous study we proposed a mechanism whereby LC-CoA competes with $PIP_2$ for the same binding site, but that LC-CoA binding does not activate the channel, thereby inhibiting channel activity by displacement of $PIP_2$ (Rapedius et al., 2005). This hypothesis has important structural implications for the mechanism of $PIP_2$ activation in Kir channels as it suggests that phosphoinositides and LC-CoA bind to the closed state of the channel. This idea is different to some previously held views about $PIP_2$ activation (as depicted in Fig. 1 B), which suggested that opening of the channel is achieved by a movement of the positively charged cytoplasmic domains toward the membrane that is driven by electrostatic attractions between the phosphoinositide binding site and membrane bound $PIP_2$ (Fan and Makielski, 1997; Enkvetchakul and Nichols, 2003). If this were the case then the simple electrostatic interaction between the binding site and $PI(3,4)P_2$ or LC-CoA should also be able to stabilize the open state in all Kir channels.

These findings argue for a relatively nonspecific electrostatic interaction between all phosphoinositides (or LC-CoA) and the binding site on the channel and suggest that specificity is achieved (at least in part) in more subtle structural changes subsequent to binding. In this model the binding of $PIP_2$ is capable of inducing pore opening, but the binding of either $PI(3,4)P_2$ or LC-CoA is not (as depicted in Fig. 1 E). Indeed, point mutations in Kir2.1 have been found that allow LC-CoA to activate the channel probably by permitting those specific interactions in the transduction mechanism required to induce channel opening (Rohacs et al., 2003).

To understand this in structural terms will ultimately require a high resolution structure of a eukaryotic Kir channel with different phosphoinositides (or LC-CoA) bound, a goal that may not be such a dream given the recent high-resolution structures of KirBac/Kir3.1 and Kv2.1/Kv2.1 chimeras produced by the MacKinnon laboratory that resolve some bound lipids and detergents, although not yet $PIP_2$ (Long et al., 2007; Nishida et al., 2007). However, recently, computational ligand docking studies have been used to define the location of $PIP_2$ and the $PIP_2$ binding site in a homology model of Kir6.2 (Haider et al., 2007). This site appears to satisfy most of the available functional data and also allows the fatty acids to reside in the membrane (Fig. 1 A). This latter point is important because the Kir channel homology models are all in the closed state and therefore suggest that it is not necessary for the phosphoinositide binding site to translocate significantly toward the membrane before binding can occur. This is therefore consistent with the proposed hypothesis in which $PI(3,4)P_2$ and LC-CoA can bind to the closed state of the channel. Furthermore, even though some of the highly conserved positive charges found within the $PIP_2$ binding site are missing in bacterial Kir channels, these channels exhibit inhibition by $PIP_2$, demonstrating that the KirBac closed state can bind $PIP_2$ (Enkvetchakul et al., 2005).

Kir channels also differ markedly in their apparent $PIP_2$ affinity as determined by their sensitivity to inhibition by various $PIP_2$ complexing agents (e.g., neomycin, polylysine, $PIP_2$ antibodies) (Huang et al., 1998; Rohacs et al., 1999, 2003). For example, Kir4.1 and Kir2.1 show very low sensitivity to neomycin (i.e., they have a high $PIP_2$ affinity), Kir1.1, Kir2.2, and $K_{ATP}$ (Kir6.2/SUR) show an intermediate sensitivity (i.e., intermediate $PIP_2$ affinity), whereas the G protein–regulated Kir3.x channels and heteromeric Kir4.1/Kir5.1 channels display a high sensitivity (i.e., they have a lower $PIP_2$ affinity) (Rapedius et al., 2007a,b). The molecular basis of these differences is currently unknown and might not be determined solely by differences in $PIP_2$ binding affinity, but also by the equilibrium between the open and closed state that is shifted toward the open state by $PIP_2$ binding (as implied by the term apparent $PIP_2$ affinity).

### From the Kinetics of $PIP_2$ Gating to the Structural Mechanism of $PIP_2$ Activation

To gain insight into the mechanism of $PIP_2$ gating we recently established an assay that allowed measurement of $PIP_2$ gating kinetics using fast application of either diC8-$PIP_2$ or neomycin to inside-out membrane patches (Rapedius et al., 2007a). These measurements revealed that $PIP_2$ rapidly binds to closed Kir channels ($\tau < 300$ ms) and then induces a much slower conformational change leading to the open state of the channel (Fig. 1 E). We found that the speed of this conformational change

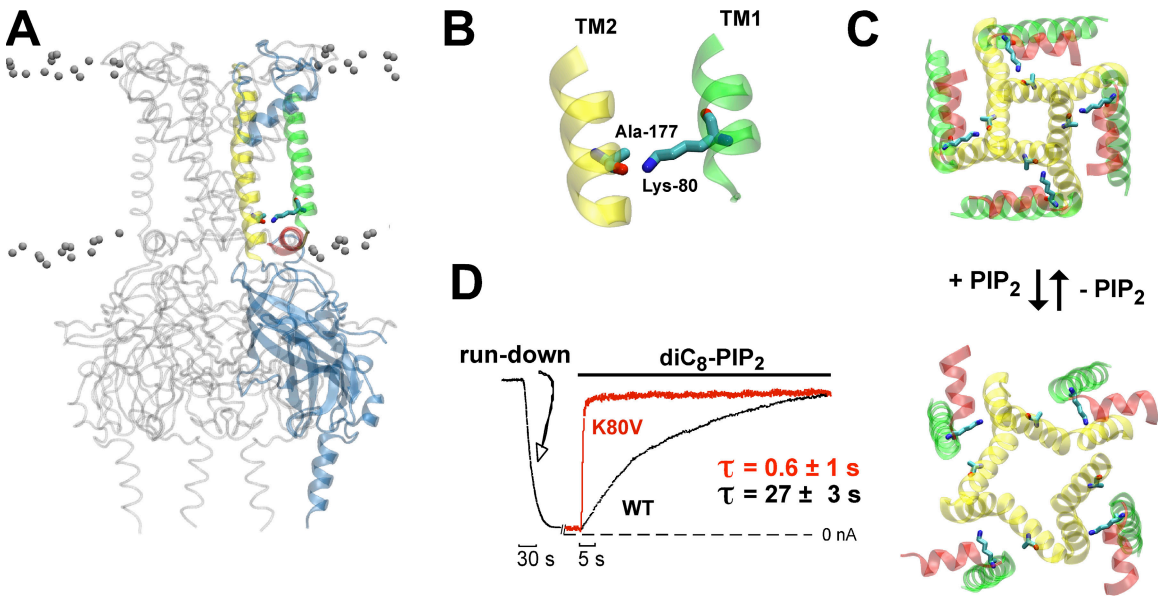


**Figure 2.** Gating model of Kir1.1. (A) Side view of a closed state homology model of Kir1.1 with one of the four subunits highlighted. The highlighted residues at the helix bundle crossing (K80 and A177) are proposed to form an H bond, which stabilizes the closed state. In this and all other panels TM1 is shown in green and TM2 in yellow with the slide helix in red. (B) An expanded view shows the proposed H bonding interaction between residues in the $-NH_3^+$ group of K80 and the backbone carbonyl group of A177. (C) A bottom up view through the pore of a closed state (top) and open state (bottom) model of Kir1.1 (for details see Rapedius et al., 2007a) illustrating a major movement of TM1 and TM1 induced by $PIP_2$ binding that would rupture the TM1–TM2 H bond. The highlighted residues are K80 and A177. (D) Rapid application of 3 μM diC8-$PIP_2$ on Kir1.1-WT and Kir1.1-K80V channels subsequent to rundown measured in inside-out patches. When H bonding at the helix bundle crossing is disrupted in Kir1.1-K80V channels, $PIP_2$ activation is much faster than in wild-type channels. A–D are modified from Rapedius et al. (2007a).

differs dramatically between Kir channels and that this depends on the presence (or absence) of a single lysine residue at the base of the first transmembrane domain (TM1). Kir channels possessing a lysine at this TM1 position (e.g., Kir1.1, Kir4.1) display slow $PIP_2$ activation, and mutation of this residue (e.g., Kir1.1[K80M], Kir4.1[K67M]) dramatically accelerates $PIP_2$ activation (~50-fold) (Fig. 2 D). In agreement with the role of this lysine residue, Kir channels lacking a lysine at this position (e.g., Kir2.1) display fast $PIP_2$ activation kinetics that are dramatically slowed upon mutation of this site to a lysine (e.g., Kir2.1[M84K]). However, this positively charged residue does not interact directly with $PIP_2$. Instead, homology modeling and molecular dynamics (MD) simulations of a Kir1.1 closed state structure suggest that the $-NH_3^+$ group of this TM1 lysine forms a hydrogen bond with the backbone carbonyl group of A177 in TM2 at the helix bundle crossing (Rapedius et al., 2007a) (Fig. 2, A and B).

A recent model of the open state KirBac3.1 suggests a large rotation and tilt of the TM1 and TM2 helices during channel opening (Domene et al., 2005; Kuo et al., 2005) that would rupture this TM1–TM2 H-bond (as depicted in Fig. 2 C), thereby explaining why this H bond determines the time course of channel opening induced by $PIP_2$ as its rupture would contribute to the activation energy of the closed to open state transition. In support of this model, a study on cysteine accessibility in Kir2.1 channels also indicates conformational changes of the pore at the helix bundle crossing during $PIP_2$ gating (Xiao et al., 2003). Taken together these results suggest that $PIP_2$ gating in Kir channels involves a major conformational change at the helix bundle crossing. However, the mechanism by which $PIP_2$ triggers this conformational change remains unknown.

There is an intriguing parallel between $PIP_2$ gating and the mechanism of pH inhibition seen in Kir channels. Kir channels with H bonding at the helix bundle crossing (e.g Kir1.1 and Kir4.1) display high sensitivity to inhibition by low intracellular pH whereas Kir channels lacking this H bonding are markedly less sensitive (Rapedius et al., 2007a). Moreover, the time course of recovery from pH inhibition corresponds strikingly well to their pH sensitivity and H bonding ability as it is fast in channels lacking H bonding and slow in channels with H bonding. This suggests that although the initial events in these gating processes are different (i.e., $PIP_2$ binding versus protonation of a currently unknown $H^+$ sensor), the structural changes at the helix bundle crossing underlying both pH gating and $PIP_2$ gating appear to be conserved. However, it is unlikely that pH inhibition simply results from promoting $PIP_2$ to unbind because the rate of $PIP_2$ activation is much slower than the recovery rate from $H^+$ inhibition. Therefore, if $H^+$ inhibition were to cause the dissociation of $PIP_2$, then the rebinding of $PIP_2$ (i.e., the $PIP_2$ activation rate)

should govern the time course of recovery from pH inhibition, which is clearly not the case (Rapedius et al., 2007a). Nevertheless, there is a general trend that Kir channels with a lower $PIP_2$ affinity show a higher pH sensitivity, indicating a possible relationship between the $PIP_2$–channel interaction and pH sensitivity (Leung et al., 2000; Schulze et al., 2003a; Du et al., 2004), but the mechanistic basis of this remains elusive.

### Physiological Implications of $PIP_2$ and LC-CoA Regulation

$PIP_2$ can be regarded as an indispensable cofactor for all eukaryotic Kir channels because no channel activity is seen in its absence. Not surprisingly therefore, inherited mutations that reduce $PIP_2$ affinity result in a variety of disease states such as Type II Bartter's Syndrome (Kir1.1), Andersen's Syndrome (Kir2.1) (Lopes et al., 2002), and congenital hyperinsulinaemia (Kir6.2) (Lin et al., 2006). This therefore raises the question as to the biological benefit of having $PIP_2$ as a cofactor. One hypothesis put forward by Hilgemann suggested that this $PIP_2$ dependence might be a mechanism to keep Kir channels inactive in intracellular membrane compartments (where $PIP_2$ levels are thought to be low) because active Kir channels might be harmful to the ion homeostasis of these compartments (Hilgemann, 2007b). Furthermore, the $PIP_2$ dependence may have allowed the evolution of mechanisms that control Kir channel activity by modulation of the interaction with $PIP_2$ (for reviews see Logothetis et al., 2007b; Xie et al., 2007) For example, G protein activation of Kir3.x channels requires $PIP_2$ and is thought to result from increasing the channel $PIP_2$ affinity from a level that is otherwise too low for the endogenous $PIP_2$ levels to support activity (Huang et al., 1998; Zhang et al., 1999). By contrast, $PIP_2$ strongly antagonizes ATP inhibition of $K_{ATP}$ channels, and vice versa (Baukrowitz et al., 1998; Shyng and Nichols, 1998). Thus, ATP inhibition can be regarded as a reduction in the apparent $PIP_2$ affinity. The molecular mechanisms behind this are still controversial and could result either (or both) from the direct displacement of $PIP_2$ by ATP (MacGregor et al., 2002; Haider et al., 2007) (the binding sites show some overlap), or an allosteric mechanism by which ATP stabilizes a conformation that prevents $PIP_2$ binding (Enkvetchakul and Nichols, 2003). Furthermore, the regulation of Kir1.1 and Kir3.x by PKA and PKC also probably involves changes in $PIP_2$ affinity (Liou et al., 1999; Zeng et al., 2003; Logothetis et al., 2007b; Xie et al., 2007). Therefore, understanding the molecular mechanisms that underlie $PIP_2$ gating may well represent the key to understanding Kir channel gating in general.

The issue of whether Kir channels are regulated by cellular phosphoinositide metabolism in vivo, and the physiological relevance of such regulation, is still controversial (Gamper and Shapiro, 2007a) due to the high $PIP_2$ affinity of many Kir channels (e.g., Kir1.1, Kir2.1, Kir4.1). For example, stimulation of the principal enzyme involved in $PIP_2$ hydrolysis (PLC), can be used as a mechanism to deplete cellular $PIP_2$ levels. However, PLC-mediated inhibition of Kir2.1 channels (which have high $PIP_2$ affinity) can only be obtained when their $PIP_2$ affinity is reduced by site-directed mutagenesis (Hardie et al., 2004). By contrast, PLC-mediated inhibition of Kir3.x channels (low intrinsic $PIP_2$ affinity) has been reported in native tissues (Cho et al., 2005) and possibly contributes to channel desensitization upon cardiac vagus stimulation by muscarinic receptors (Kobrinsky et al., 2000).

It is also still unclear as to whether such regulation occurs by a global depletion of plasma membrane $PIP_2$, or whether this occurs in microdomains (Gamper and Shapiro, 2007b; Hilgemann, 2007a; Xie et al., 2007). The latter mechanism seems more appealing because global $PIP_2$ depletion should have many potential side effects given that $PIP_2$ is involved in so many other cellular mechanisms including calcium signaling, endo-/exocytosis, organization of the cytoskeleton, and the regulation of many other ion channels and transporters. A physiological role for Kir channel regulation by other phosphoinositides such as $PI(3,4)P_2$ or $PI(3,4,5)P_3$ is rather unlikely because even when their production is stimulated (e.g., by growth hormones via PI-3 kinases) their global concentration is still very low compared with $PIP_2$ (Gamper and Shapiro, 2007a). Nevertheless, these different phosphoinositides have been mechanistically insightful in dissecting the structural basis of phosphoinositide specificity and the mechanism of $PIP_2$ activation.

LC-CoA esters are obligate intermediates of fatty acid metabolism that are formed in the cytoplasm and then shuttled into the mitochondria to fuel β-oxidation. In excised membrane patches, Kir channels can be markedly activated (or inhibited) by oleoyl-CoA at concentration in range of 100 to 1000 nM (Liu et al., 2001; Branstrom et al., 2004; Rapedius et al., 2005). However, the concentration of LC-CoA in the cytoplasm is strongly buffered by specific acyl-CoA binding proteins (Knudsen et al., 2002) and calculations suggest that the concentration of free LC-CoA is <10 nM under physiological conditions and should not exceed 200 nM even in pathophysiological conditions (Knudsen et al., 2002). This raises the question as to whether cytoplasmic levels of LC-CoA can rise sufficiently to affect Kir channels in vivo. However, studies have shown that extracellular application of oleic acids to cells can produce $K_{ATP}$ channel activation and Kir2.1 inhibition via the cytoplasmic formation of LC-CoA under conditions (e.g., high glucose) that promote LC-CoA accumulation (Branstrom et al., 2004; Shumilina et al., 2006). Such activation of $K_{ATP}$ channels in pancreatic β cells could contribute to a reduction in insulin secretion in type II diabetes patients (Riedel et al., 2003; Branstrom et al.,

2004), and the activation of cardiac $K_{ATP}$ during ischemia (which is known to promote LC-CoA accumulation) could represent a potential cardio-protective mechanism (Liu et al., 2001). Furthermore, LC-CoA activation of $K_{ATP}$ channels has been suggested to be involved in the sensing of circulating fatty acids by hypothalamic neurons (Lam et al., 2005).

A physiological relevance for the inhibitory effect of LC-CoA seen in most other Kir channels has so far yet to be established. However, a direct link between Kir channel activity and fatty acid metabolism could be of potential relevance to many tissues given the widespread expression of different Kir channels throughout the body and the general occurrence of LC-CoA in cells. Interestingly, a recent study demonstrated that the sodium–calcium exchanger, which is also regulated by $PIP_2$, displays similar regulation by LC-CoA (Riedel et al., 2006). It will therefore be interesting to see whether other $PIP_2$-regulated channels or transporters also exhibit regulation by LC-CoA.

In conclusion, it is now clear that $PIP_2$ serves as an obligate cofactor for Kir channel activity and it is difficult to examine any form of Kir channel regulation without considering the role of $PIP_2$. Work over the past decade has provided significant insight into the physiological aspects of phosphoinositide-mediated regulation and its structural mechanisms. It will also be interesting to see whether the lessons we have learned from Kir channels about, e.g., the regulation of $PIP_2$ affinity, phosphoinositide (or LC-CoA) specificity and gating efficacy, as well as the kinetics of $PIP_2$ gating can provide an insight into the molecular mechanisms by which so many diverse classes of other ion channels and transporters are regulated by phosphoinositides.

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