Phosphatidylinositol 4,5-Bisphosphate (PIP$_2$) Modulation of ATP and pH Sensitivity in Kir Channels

A TALE OF AN ACTIVE AND A SILENT PIP$_2$ SITE IN THE N TERMINUS

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Phosphatidylinositol polyphosphates (PIPs) are potent modulators of Kir channels. Previous studies have implicated basic residues in the C terminus of Kir6.2 channels as interaction sites for the PIPs. Here we examined the role of the N terminus and identified an arginine (Arg-54) as a major determinant for PIP$_2$ modulation of ATP sensitivity in K$_{ATP}$ channels. Mutation of Arg-54 to the neutral glutamine (R54Q) had no effect. These data suggest that electrostatic interactions between PIP$_2$ and Arg-54 are an essential step for the modulation of ATP sensitivity. This N-terminal PIP$_2$ site is highly conserved in Kir channels with the exception of the pH-gated channels Kir1.1, Kir4.1, and Kir5.1 that contain a neutral residue at the corresponding positions. Introduction of an arginine at this position in Kir1.1 channels rendered the N-terminal PIP$_2$ site functional largely increasing the PIP$_2$ affinity. Moreover, Kir1.1 channels lose the ability to respond to physiological changes of the intracellular pH. These results explain the need of a silent N-terminal PIP$_2$ site in pH-gated channels and highlight the N terminus as an important region for PIP$_2$ modulation of Kir channel gating.

Kir channels are a superfamily of eukaryotic channel proteins that are expressed in many tissues and responsible for important physiological processes such as cell excitability, insulin secretion, K$^+$ homeostasis, vascular tone, and regulation of the heart rate. Four subunits assemble to a channel. Each subunit contains two transmembrane segments with cytoplasmic N- and C-terminal domains and a connecting loop forming the pore (1). Some members of the Kir channel family are endowed with gating mechanisms such as ATP gating (K$_{ATP}$ channels) (2) and pH gating (Kir1.1 and Kir4.1 channels) (3). These gating mechanisms are central for the diverse functions of Kir channels in physiology and the understanding of the related pathophysiology. Kir1, Kir4, and Kir5 channels, that are predominantly expressed in epithelia, are exquisitely sensitive to changes in intracellular pH in the physiological range (3–5). This pH sensitivity is mediated by the protonation of a lysine in the N terminus (Lys-80 in Kir1.1) that induces closure of the channel’s pore by an allosteric mechanism (pH gating) (3, 6). Even small changes in the pH sensitivity can cause severe kidney defects such as the Bartter syndrome (3), highlighting the physiological importance of proper pH gating in Kir1.1 channels. Kir6 channels display a very ubiquitous expression pattern and, in coassembly with the sulfonylurea receptor (SUR),$^1$ represent the ATP-sensitive K$^+$ channels (K$_{ATP}$ channels) (7). Intracellular ATP closes K$_{ATP}$ channels by binding to the Kir6.2 subunits (ATP gating), whereas the SURs act as regulatory subunits endowing the channel with sensitivity to MgADP and pharmacological compounds. The ATP/ADP dependence of K$_{ATP}$ channels couples cell metabolism to membrane excitability, which plays an important role in the physiology of many tissues (e.g. pancreas, heart, brain) (1, 2, 8). Highly negatively charged membrane phospholipids, in particular the phosphatidylinositol polyphosphates (PIPs), such as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$), were found to interact with Kir channels, and in general they stabilize the open state of the channel (9–14). In addition, PIPs were shown to interfere with the different gating mechanisms of Kir channels. A recent report indicated modulation of pH sensitivity of Kir1.1 channels by PIP$_2$ since a mutation in the C terminus (R188Q) that reduced PIP$_2$ binding also changed the pH sensitivity (15). Further, PIP$_2$s are effective modulators of K$_{ATP}$ channels because they reduce the sensitivity to inhibition by intracellular ATP (12, 13). The effect on ATP sensitivity is of particular physiological importance since the amount of ATP inhibition determines the activity of K$_{ATP}$ channels in cells. Moreover, regulation of PIPs levels via signal transduction pathways represents an effective means to regulate K$_{ATP}$ channels by various receptors (12, 13, 16–18). PIP$_2$ was shown to interact with basic residues in the C terminus of Kir6.2. For Kir6.2/SUR channels the basic residues cluster in two regions of the C terminus (176–222 and 303–314) (11–13, 19). Further, two regions in the cytoplasmic C terminus (20, 21) and one region in the N terminus (21) have been identified where mutations markedly reduce ATP sensitivity. Intriguingly, the two C-terminal regions (near 182–185 and 333–338) are in proximity (at least in primary sequence) to the regions that are implicated in PIP$_2$ binding (176–222 and 303–314); however, the mechanistic basis of this coincidence is not clear. The region near Arg-50 in the N terminus has been implicated in ATP inhibition (21), and we mutated, therefore, basic residues in this region (Lys-47/Lys-67) to screen for PIP$_2$ binding sites. This approach identified Arg-54 as an important determinant for PIP$_2$ binding in K$_{ATP}$ channels that appeared to be vital for mediating the

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antagonizing effect of PIP$_2$ on ATP inhibition. Further, we demonstrated that the N-terminal PIP$_2$ site is silent in pH-gated Kir channels (e.g. Kir1.1) because they lack a positively charged residue at the relevant position. Introduction of a positively charged residue (e.g. arginine) largely increased PIP$_2$ binding and, more importantly, impaired pH gating explaining the need of a silent N-terminal PIP$_2$ site for pH-gated Kir channels.

**MATERIALS AND METHODS**

*Mutagenesis, cRNA Synthesis, and Oocyte Injection—*Murine Kir6.2, rat SUR2A, and Kir.1.1 (ROMK1) were used in this study. Site-directed mutagenesis was performed as described (22) and verified by sequencing. For oocyte expression, constructs were subcloned into the pGF expression vector (23). Capped cRNAs were synthesized in vitro using SP6 polymerase (Promega, Heidelberg, Germany) and stored in stock solutions at −70 °C. *Xenopus* oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for SUR2A and Kir6.2 subunits was removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for SUR2A and Kir6.2 subunits was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) and incubated at 19 °C for 1–2 days and defolliculated prior to use.

*Electrophysiology—*Giant patch recordings (22) in inside-out configuration under voltage-clamp conditions were made at room temperature (−23 °C) 4–7 days after cRNA injection. Neomycin and ATP were purchased from Sigma, l-s-Phosphatidyl-t-myo-inositol 4,5-phosphate (PIP$_2$ from bovine brain) was purchased from Roche Molecular Biochemicals, stored as stocks (1 mM) at −80 mV (inward currents shown as upward deflection), patch excision, and application of 10 μM PIP$_2$ are indicated. C, bars represent the fold change of current amplitude ± S.E. (n > 3) upon application of 10 μM PIP$_2$ for 45 s.

**RESULTS**

**Arg-54 in the N Terminus of Kir6.2 Is a Major Determinant for PIP$_2$ Binding**—The region near Arg-54 (Lys-47–Lys-67) was screened for residues that might contribute electrostatically to the binding of PIP$_2$ or ATP (Fig. 1A). Basic residues in this region (Lys-47, Arg-50, Arg-54, Lys-67) were mutated to the negatively charged amino acid glutamate. This charge-reversing substitution should reduce the binding of PIP$_2$ or ATP via electrostatic repulsion if the residue is located close to the respective binding sites. We found that ATP sensitivity was reduced for R50E (as reported previously, (21)) and, surprisingly, increased for R54E, whereas K47E and K67E channels displayed ATP sensitivities similar to WT channels (see below, Fig. 3D). To assess the impact of the different mutants on PIP$_2$ binding we compared the current amplitude briefly after patch excision with that obtained after application of PIP$_2$. This type of assay has been used previously (19) and is based on the finding that PIP$_2$ increases the channel’s open probability ($P_o$). For WT channels PIP$_2$ increased the current amplitude (thus $P_o$) only marginally (Figs. 1C and 4B). This suggests that the affinity for PIP$_2$ is so high that the endogenous PIP$_2$ already maximally opens the channels. Similar results were obtained for the mutants K47E, R50E, and K67E (Fig. 1C). In distinction, R54E channels showed only little initial activity, but application of PIP$_2$ increased the current amplitude by a factor of 29 ± 9 suggesting a marked reduction in PIP$_2$ affinity (Figs. 1, B and C and 4C). To investigate whether the charge at position 54 is critical, Arg-54 was mutated to a neutral (R54Q) and to a positively charged amino acid (R54K). R54K channels showed WT behavior, whereas the current produced by R54Q channels was increased by a factor of 4.5 ± 1.5 by PIP$_2$ (Fig. 1C). The impact of mutations on PIP$_2$ modulation in the order R54E > R54Q > R54K = WT suggest electrostatic interactions between the charge at position 54 and PIP$_2$. The mutation R176A in the C terminus of Kir6.2 has been previously shown to reduce PIP$_2$ binding (11, 13) and is shown here for comparison. The current amplitude produced by R176A channels was increased by PIP$_2$ by a factor of 3.2 ± 0.5 (Fig. 1C).

**Neomycin Inhibition as an Assay for PIP$_2$ Affinity in Kir Channels**—Neomycin is a polycation that binds specifically to PIP$_2$ and for this reason has been used to determine the PIP$_2$ content in biological membranes (24). In electrophysiological experiments, neomycin was shown to reverse the effects of PIP$_2$ on $K_{ATP}$ channels causing inhibition of channel activity and reduction of ATP sensitivity (11, 25). Thus, the neomycin sensitivity of a Kir channel might be a measure of its PIP$_2$ affinity. Accordingly, channels with a high PIP$_2$ affinity are expected to be less sensitive to neomycin than those with low PIP$_2$ affinity. To test this assumption we measured neomycin inhibition of $K_{ATP}$ and Kir1.1 channels since Kir1.1 channel are thought to bind PIP$_2$ more tightly than $K_{ATP}$ channels. In good agreement, $K_{ATP}$ channels (IC$_{50}$ = 17.1 ± 2.2 μM) were about 3-fold more sensitive to neomycin than Kir1.1 channels (IC$_{50}$ = 43 ± 10 μM) (Fig. 2C). Using this assay, we tested for the PIP$_2$ affinities of the different N-terminal (K47E, R50E, R54E, R54Q) mutants and the C-terminal mutant R176A of Kir6.2. Some of the mutated channels produced only small currents (R54E, R54Q, R176A) necessitating the measurement of neomycin inhibition subsequent to application of PIP$_2$ (10 μM for 45 s) (Fig. 2A). The mutations R54E, R54Q, and R176A altered neomycin inhibition considerably: the concentration-response curves were much steeper and most of the inhibition occurred between 1 μM and 10 μM (IC$_{50}$ of about 3 μM) (Fig. 2, A and B). For K47E channels neomycin sensitivity was somewhat increased (IC$_{50}$ = 38 ± 10 μM) and for R50E channels somewhat reduced (IC$_{50}$ = 318 ± 183 μM) compared with WT channels (IC$_{50}$ = 122 ± 98 μM after PIP$_2$) (Fig. 2C), whereas the shape of the concentration-response curves was not changed. These results are qualitatively in good agreement with the findings on PIP$_2$-amplitude modulation (Fig. 1). R54E, R54Q, and R176A largely affected PIP$_2$-amplitude modulation and neomycin sensitivity,
Arg-54 Determines PIP2 Modulation of ATP Sensitivity in Kir1.1 Channels—The effect of the N-terminal mutations as well as the C-terminal mutant R176A on the PIP2 modulation of ATP inhibition was characterized by comparing the ATP sensitivity before and subsequent to application of 10 μM PIP2 for 45 s. This procedure shifted the IC50 for ATP inhibition of WT channels by a factor of 71 ± 3 (Fig. 3, B, C, and D). Similar values were obtained for K47E (shift factor: 55 ± 17), R50E (shift factor 98 ± 16), and R67E (shift factor 73 ± 15) (Fig. 3C). For R176A the shift factor was reduced to 28 ± 4 (Fig. 3C) in agreement with previous findings on this mutant (13). R54E and R54Q had by far the largest effects on the PIP2 modulation of ATP inhibition with the corresponding shift factors of 2.5 ± 0.3 and 8.2 ± 1, respectively (Fig. 3, A, B, and C). Thus, the mutations R54E and R54Q impaired the modulation of ATP sensitivity by PIP2 with R54E being more potent than R54Q. The impact of R54Q on the antagonistic effect of PIP2 on ATP inhibition is shown directly in Fig. 4A. Application of 50 μM PIP2 readily removed inhibition of WT channel produced by 1 mM ATP (n = 3, Fig. 4A). For R54Q channels even prolonged application (see time scale) of 50 μM PIP2 only marginally antagonized ATP inhibition (n = 3, Fig. 4A). Very similar results were obtained for R54E, whereas R54K showed WT behavior (data not shown). For R176A channels PIP2 modulation of ATP inhibition was significantly reduced; however, clearly less pronounced compared with R54E/R54Q channels (n = 3, Fig. 4A). Fig. 4, B and C shows the effect of PIP2 and ATP on a single WT and R54E channel. WT channels display high initial P50 (>0.8) that was only marginally increased upon application of PIP2. Given subsequent to PIP2, 100 μM ATP produced virtually no inhibition (Fig. 4B). In contrast, R54E channels have very low initial P50 (<0.05), and PIP2 largely increased the P50 to a level similar to WT channels (>0.8); however, 100 μM ATP potently inhibited channel activity (Fig. 4C) in full agreement with the macroscopic currents (Figs. 1 and 3). In summary, these results show that the mutations R54E/R54Q disrupt the ability of PIP2 to antagonize ATP inhibition.

Role of the N-terminal PIP2 Site in Kir1.1 Channels—The arginine at position 54 is highly conserved among the members of the Kir channel superfamily; however, Kir1.1, Kir4.1, and Kir1.1 possess a neutral residue at the corresponding position (Fig. 5A). These channels have in common that they are gated by intracellular protons (3–6, 26). We chose Kir1.1 to study the role of the position Ile-63 that corresponds to Arg-54 in Kir6.2. An Arginine was introduced at position 63 (I63R), and the impact on PIP2 affinity was assayed by monitoring the rundown of channel activity upon exposure to a Mg2+ containing solution (Fig. 5B). Mg2+ is thought to induce a rundown via a breakdown of PIP2 through activation of phosphatases and lipases associated with the patch (10). WT channels lost most of the channel activity within 12 min. In I63R channels rundown was markedly slower indicative of an increased PIP2 affinity (Fig. 5B). Accordingly, the neomycin inhibition of I63R channels was reduced dramatically from an IC50 of 43 ± 10 μM (WT channels) to 7.3 ± 1.7 μM (Fig. 5C). To investigate the role of position 63 for pH gating, WT and I63R channels were exposed to various pH values of the bathing solution. Acidification from 7.5 to 6.0 resulted in complete but reversible inhibition of Kir1.1 channels (Fig. 5E). Using a Hill equation the effective pKα and Hill coefficient for pH inhibition were estimated to be 6.83 ± 0.04 and 4.4 ± 0.2, respectively, in good agreement with previous reports (Fig. 5D) (3, 6). The mutation I63R largely reduced the pH sensitivity shifting the pKα to 5.77 ± 0.08 and the Hill coefficient to 2.2 ± 0.1 (Fig. 5, D and F). If this shift in pH sensitivity was caused by an increased binding of PIP2, then application of PIP2 should cause a similar shift in pH sensitivity for WT channels. However, application of PIP2 had no significant effect on pH inhibition (Fig. 5D) suggesting that PIP2 binding was already sat-
similar results were obtained in two further experiments.

The mutation R54E resulted in K\textsubscript{ATP}
Kir6.2/SUR Channels — increased by addition of exogenous PIP\textsubscript{2} (PIP\textsubscript{2}-amplitude modulation). PIP\textsubscript{2} had little effect. The PIP\textsubscript{2} affinity of the different mutant channels an intermediate, and R54E the lowest PIP\textsubscript{2} affinity. WT and R54K channels having the highest affinity, R54Q, and R54E/R54Q dramatically reduced the effect of PIP\textsubscript{2} on ATP inhibition with R54E being more potent than R54Q. The results are in excellent agreement with previous work (11, 13, 19). The effect of R176A on PIP\textsubscript{2}-amplitude modulation was comparable to R54Q (Fig. 1C) suggesting similar importance for the binding of PIP\textsubscript{2} to K\textsubscript{ATP} channels. PIP\textsubscript{2} binding of WT and R176A channels showed a markedly increased sensitivity to neomycin compared with WT, K47E, and R50E channels. These results further substantiate the view that Arg-54 directly contributes to PIP\textsubscript{2} binding.

Arg-54 Is an N-terminal Determinant for PIP\textsubscript{2} Binding in Kir6.2/SUR Channels — The mutation R54E resulted in K\textsubscript{ATP} channels with very low P\textsubscript{o} upon patch excision that was largely increased by addition of exogenous PIP\textsubscript{2} (PIP\textsubscript{2}-amplitude modulation) consistent with a reduced PIP\textsubscript{2} affinity. In contrast, WT channels as well as the mutants K47E, R50E, and K67E showed nearly maximal P\textsubscript{o} upon patch excision, and exogenous PIP\textsubscript{2} had little effect. The PIP\textsubscript{2} affinity of the different mutant channels clearly correlated with the charge at position 54 with WT and R54K channels having the highest affinity, R54Q channels an intermediate, and R54E the lowest PIP\textsubscript{2} affinity. These results strongly suggest electrostatic interaction between Arg-54 and PIP\textsubscript{2}. Previous work has identified several residues in the C terminus of Kir6.2 as determinants for PIP\textsubscript{2} binding, e.g. R176A was shown to reduce PIP\textsubscript{2} binding (11, 13, 19).
with those on PIP_2-amplitude modulation and show that the charge at position Arg-54 determines the effect of PIP_2 on \( P_o \) and on ATP sensitivity. 

On the Mechanism of PIP_2 Modulation of ATP Inhibition—As pointed out in the introduction, regions implicated to be important for ATP inhibition and PIP_2 binding appear to coincide (at least in primary sequence) in the C terminus of Kir6.2 (19–21). This tendency seems to be even more striking with the identification of Arg-54 as a determinant of PIP_2 binding in the N terminus since Arg-50 is an important determinant of ATP inhibition (21). On the mechanistic basis of this finding two explanations come to mind. First, ATP and PIP_2 might bind to overlapping sites, and secondly, PIP_2 modulates ATP inhibition allosterically by interaction with basic residues in regions that are critical for the gating mechanism that links ATP binding to channel closure. Both alternatives have been put forward recently (27–29). As an argument against physically overlapping binding sites it has been pointed out that mutations in the C terminus that affected PIP_2 binding in the most cases did not change ATP inhibition (19). This holds valid also for the N terminus. R50E markedly reduced ATP sensitivity but had no effect on PIP_2 modulation of ATP inhibition. On the contrary, R54E largely reduced the effect of PIP_2 on ATP inhibition but had no direct effect on ATP sensitivity. Assuming electrostatic repulsion between R54E and PIP_2 it is rather unlikely for ATP binding to occur in close proximity to position 54 without sensing the charge at this position. These results argue against overlapping binding sites for PIP_2 and ATP in the N terminus and favor an allosteric mechanism. In other words the interaction of PIP_2 with Arg-54 appears to disrupt the mechanism that allows ATP to induce channel inhibition. Accordingly, disabling the N-terminal PIP_2 site is expected to reduce the ability of PIP_2 to modulate the ATP sensitivity as seen with R54E/R54Q channels. The identification of several PIP_2 sites in N and C terminus raises the question whether the sites are functionally equivalent. Are there PIP_2 sites that preferentially affect ATP inhibition and others that are more important for control of e.g. open-state stability? Comparing R176A and R54Q suggests such functional heterogeneity. Both mutations increased PIP_2-amplitude modulation to a similar extent (Fig. 1C) suggesting that Arg-54 and Arg-176 contribute about equally to the effect of PIP_2 on open-state stability. However, Arg-176 contributed obviously less to the antagonizing effect of PIP_2 on ATP inhibition compared with Arg-54 (Figs. 3C and 4A). Moreover, none of the other potential PIP_2 binding sites in the C terminus identified so far appeared to be very critical for the PIP_2 modulation of ATP sensitivity. Mutations at these positions produced significant effects on PIP_2-amplitude modulation, but no marked effects on the ability of PIP_2 to shift ATP sensitivity have been observed (19). These findings suggest a pivotal role of Arg-54 for mediating the antagonizing effect of PIP_2 on ATP inhibition.

Role of the N-terminal PIP_2 Site for pH Gating in Kir Channels—The arginine at position 54 is highly conserved among the Kir channel superfamily pointing to a general role for PIP_2 binding in Kir channels. Indeed, a recent paper demonstrated that this arginine also contributes to PIP_2 binding in Kir2.1 channels (30). Intriguingly, Kir1.1, Kir4.1, and Kir5.1 present with a neutral residue at the corresponding position. A distinctive property of these Kir channels is a strong effect of intracellular protons on the open probability of the channel. This pH gating is mediated by a lysine residue (Lys-80 in Kir1.1) in the N terminus that serves as a pH sensor and is lacking in other Kir channels (3, 6). Protonation of the pH sensor causes reversible inhibition of channel activity in the physiological range. The unusual acidic \( \Delta pK_a \) (~6.8) of the lysine is thought to come about from its proximity to two arginines in the N and C terminus of the same subunit forming a “Arg-Lys-Arg triad” that shifts the \( \Delta pK_a \) of the lysine into the physiological range via electrostatic interactions (3).

It has been proposed that PIP_2 binding to Kir1.1 alters the \( \Delta pK_a \) for pH gating (15). Thus, we tested whether the absence of the N-terminal PIP_2 site might be a prerequisite for Kir1.1 channels to operate in the physiological pH range (6.8–7.5). We observed, indeed, that introduction of an arginine at the N-terminal PIP_2 site largely increased the PIP_2 affinity of Kir1.1 channels and shifted the \( \Delta pK_a \) for pH inhibition far out of the physiological range (\( \Delta pK_a \) for I63R ~ 5.8). It has been shown that reduction in PIP_2 binding can shift the effective \( \Delta pK_a \) for pH gating in Kir1.1 channels to more alkaline values. This was based on the finding that low PIP_2 concentrations in the membrane or reduction in PIP_2 binding, as seen with a mutant channel (R188Q), increased the pH sensitivity (15). We found that PIP_2 binding is already saturated for WT Kir1.1 channels since addition of exogenous PIP_2 caused no additional shift in the \( \Delta pK_a \). This outcome does not conflict with previous findings (15); however, it suggests that an increase in PIP_2 affinity (e.g. I63R) should not alter pH gating and, thus, should not account for the effect of I63R on pH gating. PIP_2 interaction with the N-terminal site might either reduce H⁺ binding to the pH sensor thus shifting the \( \Delta pK_a \) of lysine 80 (e.g. though a change of the micro environment of Lys-80) or disturb the transduction mechanism allowing protonation of the pH sensor to power channel closure. Indeed, the reduction of the Hill coefficient for I63R (WT = 4.2 ± 0.3 and I63R = 2.2 ± 0.1, Fig. 5D) indicate reduced coupling between the pH sensor and the gate that controls channel activity. In conclusion, ATP gating in R176A channels and pH gating in Kir1.1 are controlled by a N-terminal PIP_2 site. While for \( \Delta K_{ATP} \) channels this site is necessary to allow potent modulation of ATP sensitivity by PIP_2, a silent N-terminal PIP_2 site is a prerequisite for intact pH gating in Kir1.1 channels. Even subtle changes in the pH sensitivity of Kir1.1 channels cause severe kidney defects as found in patients with the antenatal Bartter syndrome (3). Thus, a high evolutionary pressure to preserve a neutral residue at the N-terminal PIP_2 site is expected and consistent with the absence of a positively charged residue in all pH-gated Kir channels.

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