THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 12, Issue of March 21, pp. 10500–10505, 2003
Printed in U.S.A.


# 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*

Received for publication, August 17, 2002, and in revised form, December 2, 2002
Published, JBC Papers in Press, January 4, 2003, DOI 10.1074/jbc.M208413200

**Dirk Schulze‡, Tobias Krauter‡§, Hariolf Fritzenschaft, Malle Soom, and Thomas Baukrowitz¶**

*From the Institute of Physiology II, Friedrich Schiller University Jena, Teichgraben 8, 07740 Jena, Germany*

**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) and, in particular, to the negatively charged glutamate (R54E) impaired $PIP_2$ modulation of ATP inhibition, while mutation to lysine (R54K) 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 ($PIP_2$), 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, PIPs 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

* This work was supported by Grant Ba 1793 from the Deutsche Forschungsgemeinschaft (to T. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Both authors contributed equally to this work.
§ Present address: flyion GmbH, Waldhäuserstrasse 34, 72076 Tübingen, Germany.
¶ To whom correspondence should be addressed. Tel.: 0049-3641-938860; Fax: 0049-3641-933202; E-mail: thbau@mti-n.uni-jena.de.

[1] The abbreviations used are: SUR, sulfonylurea; PIP, phosphatidylinositol polyphosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; WT, wild type.

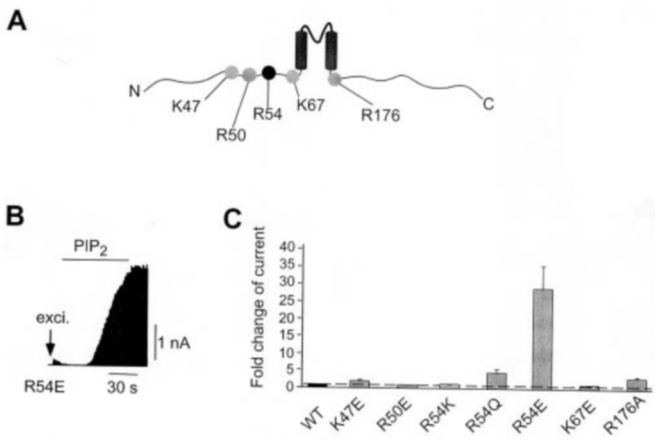



FIG. 1. **Effect of $PIP_2$ on the amplitude of currents mediated by Kir6.2/SUR2A channels and mutants expressed in *Xenopus* oocytes and studied in giant inside out patches.** The same protocol applies to all following experiments (Fig. 1–Fig. 5). *A*, schematic model of a Kir6.2 subunit residues of interest are *highlighted*. *B*, R54E currents measured at −80 mV (inward currents shown as upward deflection), patch excision, and application of 10 $\mu$M $PIP_2$ are indicated. *C*, *bars* represent the fold change of current amplitude ± S.E. ($n > 3$) upon application of 10 $\mu$M $PIP_2$ for 45 s.

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 Oocytes 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 pBF 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 injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) and incubated at 19 °C for 1–3 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) 3–7 days after cRNA injection. Neomycin and ATP were purchased from Sigma. L-a-Phosphatidyl-D-myo-inositol-4,5-phosphate ($PIP_2$ from bovine brain) was purchased from Roche Molecular Biochemicals, stored as stocks (1 mM) at −20 °C, diluted in $K_{int}$ solution to final concentrations, sonicated for 35 min, and used within 6 h. Pipettes used were made from thick-walled borosilicate glass, had resistances of 0.2–0.4 MΩ (tip diameter of 20–30 $\mu$m), and were filled with (in mM, pH adjusted to 7.2 with KOH) 120 KCl, 10 HEPES and 1.8 $CaCl_2$. Currents were recorded with an EPC9 amplifier (HEKA electronics, Lamprecht, Germany) and sampled at 1 kHz with analog filter set to 3 kHz. Solutions were applied to the cytoplasmic side of excised patches via a multibarrel pipette and had the following composition in mM ($K_{int}$): 100 KCl, 10 HEPES, 2 $K_2$EGTA (total $K^+$ concentration was 120 mM, pH adjusted to 7.2 with KOH). Computational work was done on Macintosh G4 using commercial software (IGOR, WaveMetrics) and Excel 2001 (Microsoft).

## RESULTS

*Arg-54 in the N Terminus of Kir6.2 Is a Major Determinant for $PIP_2$ Binding*—The region near Arg-50 (Lys-47–Lys-67) was screened for residues that might contribute electrostatically to the binding of $PIP_2$ or ATP (Fig. 1*A*). 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. 3*D*). 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. 1*C* and 4*B*). This suggests that the affinity for PIPs 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. 1*C*). 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 4*C*). 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. 1*C*). 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. 1*C*).

*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 $\mu$M) were about 3-fold more sensitive to neomycin than Kir1.1 channels ($IC_{50}$ = 43 ± 10 $\mu$M) (Fig. 2*C*). 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 $\mu$M for 45 s) (Fig. 2*A*). 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 $\mu$m and 10 $\mu$M ($IC_{50}$ of about 3 $\mu$M) (Fig. 2, *A* and *B*). For K47E channels neomycin sensitivity was somewhat increased ($IC_{50}$ = 38 ± 10 $\mu$M) and for R50E channels somewhat reduced ($IC_{50}$ = 318 ± 183 $\mu$M) compared with WT channels ($IC_{50}$ = 122 ± 98 $\mu$M after $PIP_2$) (Fig. 2*C*), 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,

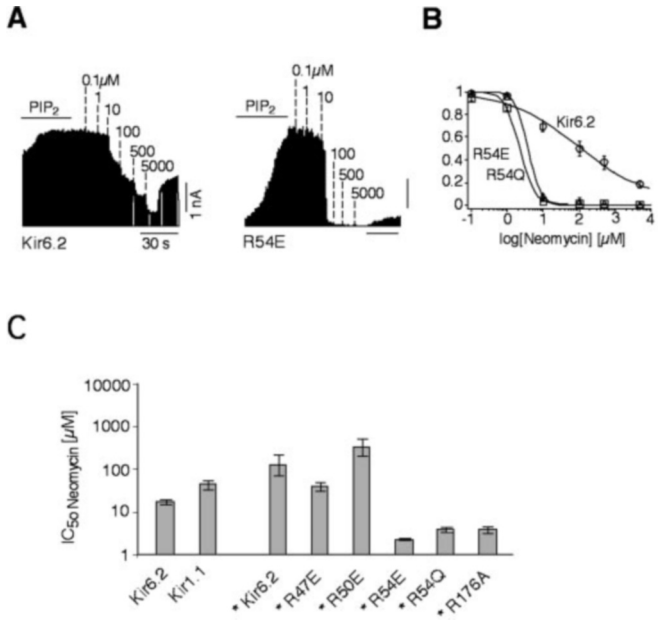
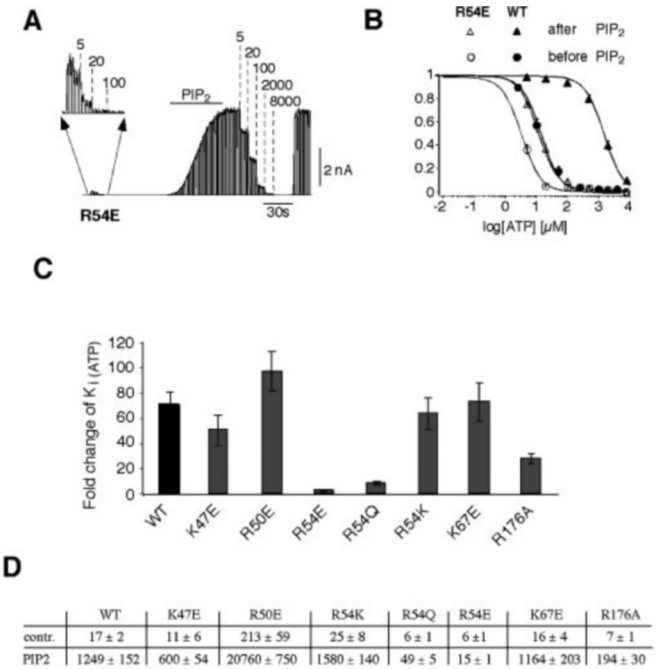



FIG. 2. **Neomycin inhibition of Kir1.1, Kir6.2, and mutant channels in inside out patches.** *A*, neomycin inhibition subsequent to application of 10 $\mu$M $PIP_2$ for 45 s; neomycin concentration as indicated. *B*, from data such as shown in *A* concentration-response curves (CV curves) were obtained and fitted to a standard Hill equation; *error bars* represent ± S.E. *C*, from CV curves as in *B* the $IC_{50}$ values were determined and plotted as bar with ± S.E. *Stars* indicate that the $IC_{50}$ values were determined subsequent to application of 10 $\mu$M $PIP_2$ for 45 s.




| | WT | K47E | R50E | R54K | R54Q | R54E | K67E | R176A |
|---|---|---|---|---|---|---|---|---|
| contr. | 17 ± 2 | 11 ± 6 | 213 ± 59 | 25 ± 8 | 6 ± 1 | 6 ±1 | 16 ± 4 | 7 ± 1 |
| PIP2 | 1249 ± 152 | 600 ± 54 | 20760 ± 750 | 1580 ± 140 | 49 ± 5 | 15 ± 1 | 1164 ± 203 | 194 ± 30 |

FIG. 3. **Effects of Kir6.2 mutations on $PIP_2$ modulation of ATP sensitivity.** *A*, R54E currents with applications of 10 $\mu$M $PIP_2$ and various ATP concentrations as indicated. *B*, from data such as in *A* CV curves were obtained and fitted to a standard Hill equation (13); *error bars* indicate ± S.E. *C*, from CV curves in *B* $IC_{50}$ values were determined and plotted as bars with ± S.E. *D*, table with values of the $IC_{50}$ values before (*contr.*) and after application of 10 $\mu$M $PIP_2$ for 45 s.

whereas K47E and R50E channels showed basically WT behavior.

*Arg-54 Determines $PIP_2$ Modulation of ATP Sensitivity in $K_{ATP}$ Channels*—The effect of the N-terminal mutations as well as the C-terminal mutant R176A on the $PIP_2$ modulation of ATP inhibition was characterized by comparing the ATP sensitivity before and subsequent to application of 10 $\mu$M $PIP_2$ for 45 s. This procedure shifted the $IC_{50}$ 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 K67E (shift factor 73 ± 15) (Fig. 3*C*). For R176A the shift factor was reduced to 28 ± 4 (Fig. 3*C*) in agreement with previous findings on this mutant (13). R54E and R54Q had by far the largest effects on the $PIP_2$ 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 $PIP_2$ with R54E being more potent than R54Q. The impact of R54Q on the antagonistic effect of $PIP_2$ on ATP inhibition is shown directly in Fig. 4*A*. Application of 50 $\mu$M $PIP_2$ readily removed inhibition of WT channel produced by 1 mM ATP ($n$ = 3, Fig. 4*A*). For R54Q channels even prolonged application (see time scale) of 50 $\mu$M $PIP_2$ only marginally antagonized ATP inhibition ($n$ = 3, Fig. 4*A*). Very similar results were obtained for R54E, whereas R54K showed WT behavior (data not shown). For R176A channels $PIP_2$ modulation of ATP inhibition was significantly reduced; however, clearly less pronounced compared with R54E/R54Q channels ($n$ = 3, Fig. 4*A*). Fig. 4, *B* and *C* shows the effect of $PIP_2$ and ATP on a single WT and R54E channel. WT channels display high initial $P_o$ (>0.8) that was only marginally increased upon application of $PIP_2$. Given subsequent to $PIP_2$, 100 $\mu$M ATP produced virtually no inhibition (Fig. 4*B*). In contrast, R54E channels have very low initial $P_o$ (<0.05), and $PIP_2$ largely increased the $P_o$ to a level similar to WT channels (>0.8); however, 100 $\mu$M ATP potently inhibited channel activity (Fig. 4*C*) 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 $PIP_2$ to antagonize ATP inhibition.

*Role of the N-terminal $PIP_2$ 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 Kir5.1 possess a neutral residue at the corresponding position (Fig. 5*A*). 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 $PIP_2$ affinity was assayed by monitoring the run down of channel activity upon exposure to a $Mg^{2+}$-containing solution (Fig. 5*B*). $Mg^{2+}$ is thought to induce a run down via a breakdown of $PIP_2$ though activation of phosphatases and lipases associated with the patch (10). WT channels lost most of the channel activity within 12 min. In I63R channels run down was markedly slower indicative of an increased $PIP_2$ affinity (Fig. 5*B*). Accordingly, the neomycin inhibition of I63R channels was reduced dramatically from an $IC_{50}$ of 43 ± 10 $\mu$M (WT channels) to 7.3 ± 1.7 mM (Fig. 5*C*).

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. 5*E*). Using a Hill equation the effective $pK_a$ 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. 5*D*) (3, 6). The mutation I63R largely reduced the pH sensitivity shifting the $pK_a$ 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 $PIP_2$, then application of $PIP_2$ should cause a similar shift in pH sensitivity for WT channels. However, application of $PIP_2$ had no significant effect on pH inhibition (Fig. 5*D*) suggesting that $PIP_2$ binding was already sat-

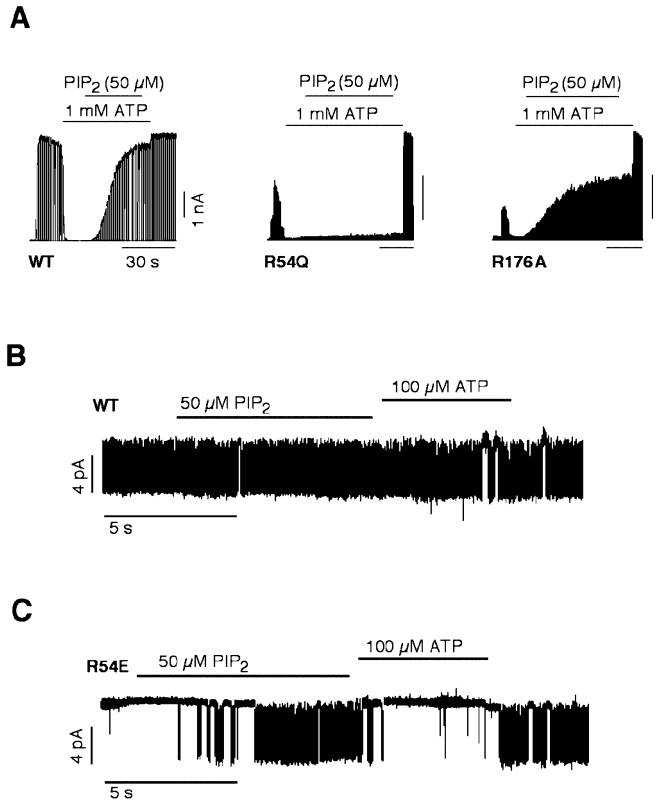



FIG. 4. **Antagonism of ATP inhibition by $PIP_2$ for WT and mutant channels.** *A*, for WT, R54Q, and R176A currents the effect of $PIP_2$ and ATP is shown with concentrations as indicated. Similar results were obtained in at least two further experiments. *B*, single channel recordings for WT and R54E channels measured at −80 mV and filtered at 2 kHz. Application of $PIP_2$ and ATP as indicated. From the last 3 s before ATP application the $P_o$ was estimated to be $> 0.8 < 0.9$ for WT and R54E. $P_o$ was calculated from amplitude histograms (data not shown). The $P_o$ of the R54E channels before $PIP_2$ was below 0.05. Similar results were obtained in two further experiments.

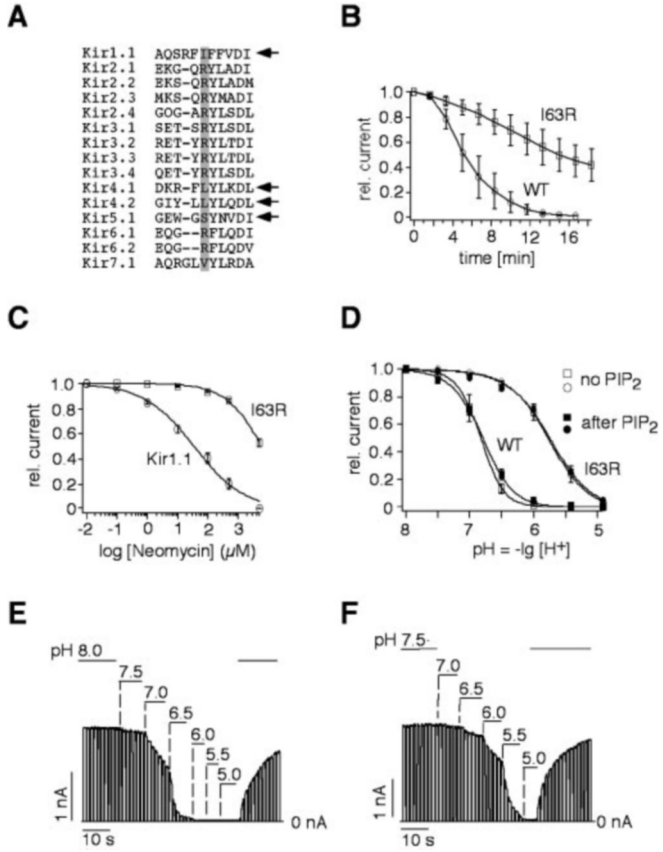



FIG. 5. **Effects of I63R on $PIP_2$ affinity and pH sensitivity of Kir1.1 channels.** *A*, sequence alignment of various Kir channels for the region near Arg-54 in Kir6.2; pH-gated channels are indicated with *arrows* (Alignment was done with the commercial software DNA-Star). *B*, time course of channel run down for WT and I63R channels induced by application of 2 mM $Mg^{2+}$; data points are mean ± S.E. *C*, neomycin inhibition of WT and I63R channels. From experiments such as in Fig. 2*A* CV curves were obtained and fitted to a standard Hill equation; *error bars* indicate ± S.E. The $IC_{50}$ values for WT and I63R were 43 ± 10 μM and 7.3 ± 1.7 mM, respectively. *E*, currents from WT and I63R channels (*F*) at the pH values indicated. *D*, from recordings such as in *E* and *F* CV-curves were obtained and fitted to a standard Hill equation (*open symbols*); *error bars* indicate ± S.E. $pK_a$ and Hill coefficient were 6.8 ± 0.02 and 4.2 ± 0.3 for WT and 5.77 ± 0.08 and 2.2 ± 0.1 for I63R channels. *Filled symbols* represent CV curves subsequent to application of 20 μM $PIP_2$ for 30 s; $pK_a$ and Hill coefficient were 6.79 ± 001 and 2.7 ± 0.3 for WT and 5.75 ± 0.04 and 1.8 ± 0.1 for I63R channels.

urated before the addition of exogenous $PIP_2$. These results point to an effect of I63R on pH gating in Kir1.1 channels that is distinct to the effect on $PIP_2$ affinity (see "Discussion").

## DISCUSSION

We screened the proximal N terminus for residues that contribute electrostatically to the binding of $PIP_2$ or ATP by substituting basic residues by the negatively charged glutamate (K47E, R50E, R54E, K67E). Two of these mutations altered ATP inhibition markedly. R50E reduced the ATP sensitivity about 12-fold, whereas R54E increased the sensitivity about 3-fold. However, neither Arg-50 nor Arg-54 are likely to contribute to ATP binding directly as detailed below.

*Arg-54 Is an N-terminal Determinant for $PIP_2$ Binding in Kir6.2/SUR Channels*—The mutation R54E resulted in $K_{ATP}$ channels with very low $P_o$ upon patch excision that was largely increased by addition of exogenous $PIP_2$ ($PIP_2$-amplitude modulation) consistent with a reduced $PIP_2$ affinity. In contrast, WT channels as well as the mutants K47E, R50E, and K67E showed nearly maximal $P_o$ upon patch excision, and exogenous $PIP_2$ had little effect. The $PIP_2$ 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 $PIP_2$ affinity. These results strongly suggest electrostatic interaction between Arg-54 and $PIP_2$. Previous work has identified several residues in the C terminus of Kir6.2 as determinants for $PIP_2$ binding, *e.g.* R176A was shown to reduce $PIP_2$ binding (11, 13, 19). The effect of R176A on $PIP_2$-amplitude modulation was comparable to R54Q (Fig. 1*C*) suggesting similar importance for the binding of $PIP_2$ to $K_{ATP}$ channels. $PIP_2$ binding of WT and mutant channels was also tested using neomycin inhibition as a relative measure for the $PIP_2$ affinity. R54E, R54Q, 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 $PIP_2$ binding.

*Arg-54 Plays a Key Role for the $PIP_2$ Modulation of ATP Sensitivity in $K_{ATP}$ Channels*—The ATP sensitivity of $K_{ATP}$ channels depends on the concentration of PIPs in the membrane. Increasing concentrations of $PIP_2$ increase the $IC_{50}$ value for ATP inhibition by several orders of magnitude (12, 13), and thus, the shift in ATP sensitivity for a given increase in membrane $PIP_2$ should be related to the $PIP_2$ affinity of the channel. We found that the mutations K47E, R50E, and K67E had virtually no effect on the $PIP_2$ modulation of ATP inhibition in contrast to R54E and R54Q. R54E/R54Q dramatically reduced the effect of $PIP_2$ on ATP inhibition with R54E being more potent than R54Q. The results are in excellent agreement

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 $PIP_2$-amplidude modulation to a similar extent (Fig. 1*C*) 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. 3*C* and 4*A*). 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 $pK_a$ (≈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 $pK_a$ of the lysine into the physiological range via electrostatic interactions (3).

It has been proposed that $PIP_2$ binding to Kir1.1 alters the $pK_a$ 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 $pK_a$ for pH inhibition far out of the physiological range ($pK_a$ for I63R ≈ 5.8). It has been shown that reduction in $PIP_2$ binding can shift the effective $pK_a$ 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 $pK_a$. 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 $pK_a$ 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. 5*D*) might indicate reduced coupling between the pH sensor and the gate that controls channel activity. In conclusion, ATP gating in $K_{ATP}$ channels and pH gating in Kir1.1 are controlled by a N-terminal $PIP_2$ site. While for $K_{ATP}$ 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.

*Acknowledgments*—We appreciate the excellent technical support by Dr. Yuan Ruan and thank Dr. Klaus Benndorf and Dr. Bernd Fakler for critically reading the manuscript.

REFERENCES

1. Nichols, C. G., and Lopatin, A. N. (1997) *Annu. Rev. Physiol.* **59,** 171–191
2. Ashcroft, F. M. (1988) *Annul. Rev. Neurosci.* **11,** 97–118
3. Schulte, U., Hahn, H., Konrad, M., Jeck, N., Derst, C., Wild, K., Weidemann, S., Ruppersberg, J. P., Fakler, B., and Ludwig, J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 15298–15303
4. Tanemoto, M., Kittaka, N., Inanobe, A., and Kurachi, Y. (2000) *J. Physiol.* **525,** 587–592
5. Tucker, S. J., Imbrici, P., Salvatore, L., D'Adamo, M. C., and Pessia, M. (2000) *J. Biol. Chem.* **275,** 16404–16407
6. Fakler, B., Schultz, J. H., Yang, J., Schulte, U., Brandle, U., Zenner, H. P., Jan, L. Y., and Ruppersberg, J. P. (1996) *EMBO J.* **15,** 4093–4099
7. Inagaki, N., Gonoi, T., Clement, J. P. I., Namba, N., Inazawa, J., Gonzales, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995) *Science* **270,** 1166–1169
8. Seino, S. (1999) *Annu. Rev. Physiol.* **61,** 337–362
9. Hilgemann, D. W., and Ball, R. (1996) *Science* **273,** 956–959
10. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) *Nature* **391,** 803–806
11. Fan, Z., and Makielski, J. C. (1997) *J. Biol. Chem.* **272,** 5388–5395
12. Shyng, S. L., and Nichols, C. G. (1998) *Science* **282,** 1138–1141
13. Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S. J., Ruppersberg, J. P., and Fakler, B. (1998) *Science* **282,** 1141–1144
14. Sui, J. L., Petit-Jacques, J., and Logothetis, D. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1307–1312
15. Leung, Y. M., Zeng, W. Z., Liou, H. H., Solaro, C. R., and Huang, C. H. (2000) *J. Biol. Chem.* **275,** 10182–10189
16. Baukrowitz, T., and Fakler, B. (2000) *Biochem. Pharmacol* **60,** 735–740
17. Xie, L. H., Horie, M., and Takano, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 15292–15297
18. Spanswick, D., Smith, M. A., Groppi, V. E., Logan, S. D., and Ashford, M. L.

(1997) *Nature* **390,** 521–525
19. Shyng, S. L., Cukras, C. A., Harwood, J., and Nichols, C. G. (2000) *J. Gen. Physiol.* **116,** 599–608
20. Drain, P., Li, L., and Wang, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13953–13958
21. Tucker, S. J., Gribble, F. M., Proks, P., Trapp, S., Ryder, T. J., Haug, T., Reimann, F., and Ashcroft, F. M. (1998) *EMBO J.* **17,** 3290–3296
22. Baukrowitz, T., Tucker, S. J., Schulte, U., Benndorf, K., Ruppersberg, J. P., and Fakler, B. (1999) *EMBO J.* **18,** 847–853
23. Fakler, B., Brändle, U., Glowatzki, E., Weidemann, S., Zenner, H. P., and Ruppersberg, J. P. (1995) *Cell* **80,** 149–154
24. Arbuzova, A., Martushova, K., Hangyás-Mihályné, G., Morris, A. J., Ozaki, S., Prestwich, G. D., and McLaughlin, S. (2000) *Biochim. Biophys. Acta* **1464,** 35–48
25. Krauter, T., Ruppersberg, J. P., and Baukrowitz, T. (2001) *Mol. Pharmcol.* **59,** 1086–1093.
26. Pessia, M., Imbrici, P., D'Adamo, M. C., Salvatore, L., and Tucker, S. J. (2001) *J. Physiol.* **532,** 359–367
27. Fan, Z., and Makielski, J. C. (1999) *J. Gen. Physiol.* **114,** 251–270
28. Enkvetchakul, D., Loussouarn, G., Makhina, E., Shyng, S. L., and Nichols, C. G. (2000) *Biophys. J.* **78,** 2334–2348
29. Ribalet, B., John, S. A., and Weiss, J. N. (2000) *J. Gen. Physiol.* **116,** 391–410
30. Lopes, C. M., Zhang, H., Rohacs, T., Jin, T., Yang, J., and Logothetis, D. E. (2002) *Neuron* **34,** 933–944