

Structural Determinants of PIP₂ Regulation of Inward Rectifier K_{ATP} Channels

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ABSTRACT Phosphatidylinositol 4,5-bisphosphate (PIP₂) activates K_{ATP} and other inward rectifier (Kir) channels. To determine residues important for PIP₂ regulation, we have systematically mutated each positive charge in the COOH terminus of Kir6.2 to alanine. The effects of these mutations on channel function were examined using ⁸⁶Rb efflux assays on intact cells and inside-out patch-clamp methods. Both methods identify essentially the same basic residues in two narrow regions (176–222 and 301–314) in the COOH terminus that are important for the maintenance of channel function and interaction with PIP₂. Only one residue (R201A) simultaneously affected ATP and PIP₂ sensitivity, which is consistent with the notion that these ligands, while functionally competitive, are unlikely to bind to identical sites. Strikingly, none of 13 basic residues in the terminal portion (residues 315–390) of the COOH terminus affected channel function when neutralized. The data help to define the structural requirements for PIP₂ sensitivity of K_{ATP} channels. Moreover, the regions and residues defined in this study parallel those uncovered in recent studies of PIP₂ sensitivity in other inward rectifier channels, indicating a common structural basis for PIP₂ regulation.

KEY WORDS: potassium channel • ATP • PH domain • Kir6.2 • phospholipid

INTRODUCTION

ATP-sensitive potassium (K_{ATP})¹ channels couple cell metabolism to excitability in many tissues (Ashcroft, 1998; Nichols and Lederer, 1991). Normally, reconstitution of K_{ATP} current requires coexpression of a sulfonylurea receptor (SUR) subunit and an inward rectifier (Kir6) subunit (Aguilar-Bryan et al., 1995; Inagaki et al., 1995), assembled into an octameric complex of four SUR and four Kir6 subunits (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997). However, truncation of the Kir6.2 COOH terminus by 26 or 36 amino acids (Kir6.2[ΔC]) enables the formation of functional channels in the absence of SUR (Tucker et al., 1997). The Kir6.2 COOH terminus contains a signal (-RKR-) that prevents exit from the endoplasmic reticulum; SUR1 shields this retention signal and chaperones the complex to the plasma membrane (Zerangue et al., 1999).

ATP inhibits K_{ATP} channels with half-maximal inhibitory concentration (K_{1/2,ATP}) of ~10 μM in excised patches (Ashcroft, 1998), and MgADP antagonizes this inhibition by an action on the SUR subunit (Ashcroft, 1998; Nichols et al., 1996; Gribble et al., 1997; Shyng et

al., 1997b). Activation of K_{ATP} channels occurs under conditions where cytoplasmic [ATP] (3–5 mM) is much higher than that required to inhibit channels in excised patches (Niki et al., 1989). The physiological regulation is mediated, at least in part, by changes in the intracellular [ATP]/[ADP] ratio, since mutations in SUR1 that abolish stimulation by MgADP remain silent, even after glucose starvation (Nichols et al., 1996; Shyng et al., 1998). Negatively charged phospholipids, particularly phosphatidylinositol-4,5-bisphosphate (PIP₂), activate many, if not all, inward rectifier K channels (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Huang et al., 1998) by increasing the open state stability of the channel. In K_{ATP} channels, application of PIP₂ leads to an increase in open probability and a decrease of ATP sensitivity (Baukrowitz et al., 1998; Shyng and Nichols, 1998; Fan and Makielski, 1999). If membrane PIP₂ levels increase, K_{ATP} channels will be rendered less sensitive to ATP, providing another potential mechanism for channel activation in physiological [ATP] (Xie et al., 1999; Shyng et al., 2000).

The antagonistic effect of PIP₂ on ATP inhibition suggests that the two ligands compete functionally for interaction with the channel. The Kir6.2 subunit is primarily responsible for these interactions, since Kir6.2[ΔC] expressed without SUR1 (Tucker et al., 1997) is still inhibited by ATP and stimulated by PIP₂ (Baukrowitz et al., 1998; Enkvetchakul et al., 2000). Binding of [³²P]azido-ATP to Kir6.2 has been demonstrated (Tanabe et al., 1999) and involves residues in

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¹Abbreviations used in this paper: K_{ATP}, ATP-sensitive potassium; PH, pleckstrin homology; PIP₂, phosphatidylinositol 4,5-bisphosphate; SUR, sulfonylurea receptor.

the cytoplasmic NH₂ and COOH termini (Drain et al., 1998; Tucker et al., 1998; Koster et al., 1999; Tanabe et al., 1999). The molecular determinants of PIP₂ activation of K_{ATP} and other Kir channels remain unclear, although several conserved COOH terminus residues are involved (Baukrowitz et al., 1998; Huang et al., 1998; Shyng and Nichols, 1998; Zhang et al., 1999). The isolated COOH terminus of Kir6.2 inhibits K_{ATP} channel activity, possibly by competing for PIP₂ binding (Shyng and Nichols, 1998). The isolated COOH terminus of Kir1.1 has also been shown to bind to PIP₂, and this binding is reduced when a putative PIP₂ binding residue (K188) is neutralized (Huang et al., 1998). Mutation of the adjacent residue (R176A) in Kir6.2 dramatically reduces channel activity (Fan and Makielski, 1997), and there is a much slower and more significant response to PIP₂ (Baukrowitz et al., 1998; Shyng and Nichols, 1998), an effect which is consistent with this mutation causing reduced affinity for PIP₂.

We have now performed systematic mutagenesis and electrophysiological analysis to determine the positively charged residues in the Kir6.2 COOH terminus that are critical for PIP₂ interaction. We report multiple such residues clustered in the proximal COOH terminus, and consider the possible structural basis for channel regulation by phospholipids.

MATERIALS AND METHODS

Molecular Biology

Constructs containing point mutations were prepared by overlap extension at the junctions of the relevant residues by sequential PCR. The resulting PCR products were subcloned into the pCMV6b vector. Before transfection, the constructs were sequenced to verify the correct mutations.

Expression of K_{ATP} channels in COSm6 Cells

COSm6 cells were plated at a density of $\sim 2.5 \times 10^5$ cells per well (30-mm six-well dishes) and cultured in Dulbecco's Modified Eagle Medium plus 10 mM glucose (DMEM-HG) supplemented with 10% FCS. The next day, cells were transfected by incubation for 4 h at 37°C in DMEM medium containing 10% Nuserum, 0.4 mg/ml diethylaminoethyl-dextran, 100 μ M chloroquine, and 5 μ g each of pCMV6b-Kir6.2 or mutant isoforms, pECE-SUR1 cDNA, and pECE-GFP (green fluorescent protein). Cells were subsequently incubated for 2 min in HEPES-buffered salt solution containing 10% DMSO, and returned to DMEM-HG plus 10% FCS.

⁸⁶Rb⁺ Efflux Assay

Cells were incubated for 24 h in culture medium containing ⁸⁶RbCl (1 μ Ci/ml) for 2–3 d after transfection. Before measurement of Rb efflux, cells were incubated for 30 min at 25°C in Krebs' Ringer solution, with metabolic inhibitors (2.5 μ g/ml oligomycin plus 1mM 2-deoxy-D-glucose). At selected time points, the solution was aspirated from the cells and replaced with fresh solution. At the end of the 40-min period, cells were lysed in 2% SDS-Ringer's solution. The ⁸⁶Rb⁺ in the aspirated solution and the cell lysates were counted. The percent efflux at each time point

was calculated as the cumulative counts in the aspirated solution divided by the total counts from the solutions and the cell lysates.

Patch-Clamp Measurements

Patch-clamp experiments were made at room temperature in a chamber that allowed rapid exchange of bathing solution. Micropipettes were pulled from thin-walled glass (WPI Inc.) on a horizontal puller (Sutter Instrument, Co.). Electrode resistance was typically 0.5–1 M Ω when filled with K-INT solution (below). Inside-out patches were voltage-clamped with an Axopatch 1B amplifier (Axon Inc.). The standard bath (intracellular) and pipette (extracellular) solution (K-INT) had the following composition: 140 mM KCl, 10 mM K-HEPES, and 1 mM K-EGTA, pH 7.3. PIP₂ was bath-sonicated in ice for 30 min before use. ATP was added as the potassium salt. All currents were measured at a membrane potential of –50 mV (pipette voltage = +50 mV), and inward currents at this voltage are shown as upward deflections. Data were filtered at 0.5–3 kHz, digitized at 22 kHz (Neuro-corder; Neurodata), and stored on videotape. Experiments were replayed onto a chart recorder or digitized into a microcomputer using Axotape software (Axon Inc.). Offline analysis was performed using Microsoft Excel programs. Wherever possible, data are presented as mean \pm SEM. Microsoft Solver was used to fit data by a least-square algorithm.

Interpretation of PIP₂ Response Data

Wild-type K_{ATP} (Kir6.2 + SUR1) channels have an intrinsic open probability in the absence of ATP ($P_{o,zero}$) of ~ 0.4 and are inhibited by ATP with $K_{1/2,ATP}$ of ~ 10 μ M (Inagaki et al., 1995; Enkvetchakul et al., 2000). Many mutations of Kir6.2, or exposure to cytoplasmic PIP₂, cause changes in $P_{o,zero}$ and ATP sensitivity. In almost all cases, $P_{o,zero}$ and $K_{1/2,ATP}$ are strongly correlated, and this can be explained by assuming that the action of ATP is on the closed channel, such that both $P_{o,zero}$ and $K_{1/2,ATP}$ are increased when open state stability is increased by addition of PIP₂ (Shyng et al., 1997a; Baukrowitz et al., 1998; Shyng and Nichols, 1998; Enkvetchakul et al., 2000). PIP₂ might bind to a specific state of the channel, and mutations could affect PIP₂ response by altering either the affinity or availability of a PIP₂ binding site. These possibilities are experimentally unresolvable. Therefore, in the present experiments, we conclude that cytoplasmic domain mutations that reduce intrinsic activity and cause a slower and greater response to PIP₂ are likely to be either involved in PIP₂ binding directly or in the translation of this binding into an effect on channel open state stability.

RESULTS

Residues Critical for Regulation of Kir6.2 by PIP₂

In the Kir6.2 COOH terminus (residues 173–390), there are 23 basic residues (Fig. 1 A), any or all of which might contribute electrostatically to PIP₂ binding. We performed alanine scanning mutagenesis of these basic residues to determine their involvement in PIP₂ sensitivity and/or ATP sensitivity. Channel activity of all mutants was initially assessed using a ⁸⁶Rb⁺ efflux assay (Fig. 1 B). In addition to the previously recognized R176 and R177 residues (Fan and Makielski, 1997; Baukrowitz et al., 1998; Shyng and Nichols, 1998), the efflux was virtually abolished in three additional mutants (R206A, K222A, and R301A), and significantly reduced in the R195A and R314A mutants. The

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1
MLSRKGI IPEEYVLR LRAEDPAEPRYRTRERRARFVSKKGN CNVAHKNI REQGRFLQDVFTTLVDL
67
KWPHLLIIFTMSFLCSWLLFAMVWVLI AFAHGD LAPGEGTNVPCVTSIHSFSSAFLFSIEV QVTTIG
133
FGGRMVTEECPLAILILIVQNI VGLMINAIMLGCIFMKTAQAH RRAETLIFS KHAVITL RHGRLCF
199
MIRVGD LR KSM ISATI HM QVVR RTTSP EGEVVP LHQVDIPMENG VGGNSIFL VAPLI IYHVIDSN
265
SPLYDLAPSDLHHHQDLEIIVILEGVVETTGITTA TSYLADEILWGQ SFVPIVAEEDGRYSVDY
331
SKFGNTIKVPTPLCTARQLDEDE RSLLDALTLASSRGLR KRSVAVAK KPKFSISPDSL S
390

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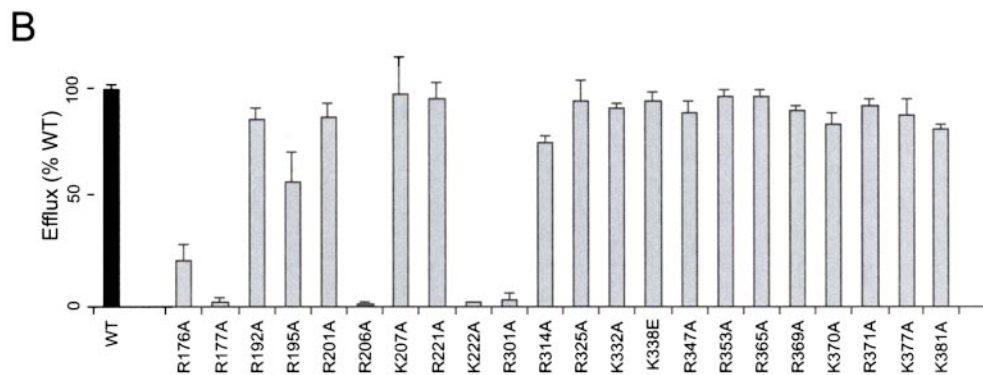


FIGURE 1. Alanine scanning mutagenesis of COOH-terminal basic residues. (A) Primary sequence of Kir6.2, highlighted to show transmembrane M1 and M2 domains together with the P-loop (gray), and regions that enclose PIP₂-sensitive residues (pink). All residues that were mutated in this study are indicated (bold), including those that alter PIP₂ response in this study (red) or ATP sensitivity (blue). Also indicated are the PIP₂-sensitive residues identified in Kir2.1/Kir3.1 by Zhang et al. (1999; orange). (B) ⁸⁶Rb efflux in 40 min from COSm6 cells cotransfected with mutant Kir6.2 subunits (as indicated) + SUR1, expressed as a percentage of the efflux from cotransfected wild-type Kir6.2 + SUR1 subunits in parallel transfections (mean ± SEM, n = 3 in each case). See MATERIALS AND METHODS for details.

affected mutants clustered in two regions of the near COOH terminus (R176–K222 and R301–R314). There was no significant reduction of efflux in any mutants downstream of R314.

Mutant channel activity was examined in more detail in inside-out membrane patches. Wild-type Kir6.2 + SUR1 channels have an intrinsic P_{o,zero} of ~0.4 (Inagaki et al., 1995; Enkvetchakul et al., 2000), and increasing PIP₂ in the membrane increases the open state stability. In wild-type channels, PIP₂ application leads to an approximately twofold increase in macroscopic channel activity as P_{o,zero} saturates at ~0.9 (Fig. 2, A and C). As considered above, mutations that reduce apparent PIP₂ affinity, either by real changes in PIP₂ binding affinity or by lowering the intrinsic stability of the open pore, will lower the intrinsic P_{o,zero}. When membrane PIP₂ is increased by cytoplasmic exposure, the increase in current in these mutants should occur more slowly than wild-type, and to a relatively greater extent.² The sensi-

tivity of mutant channels to PIP₂ stimulation was estimated from the time course (Fig. 2 B) and the extent (Fig. 2 C) of increase in relative current in response to cytoplasmic application of 5 μg/ml PIP₂. Nine mutations were identified as having altered sensitivity to PIP₂ in this assay (R176A, R177A, R195A, R206A, K222A, R301A, and R314A [identified above], plus R192A and R201A, which also show a nonsignificant reduction in Rb flux compared with wild-type). Again, there was no apparent effect of mutations downstream of R314 on PIP₂ sensitivity. The correlation between mutant effects on Rb efflux and response to PIP₂ indicates that, in each case, reduction in Rb efflux is likely due to a reduced sensitivity to the ambient phospholipid level in the intact cell.

As shown previously (Fan and Makielski, 1997; Shyng and Nichols, 1998; Baukowitz et al., 1998), the R176A mutation greatly reduces intrinsic channel activity. The response to PIP₂ is markedly slower, and the relative current increase is much greater, than wild-type (Fig. 2, B and C), which is consistent with reduced PIP₂ affinity. The R177A mutation abolishes channel activity (Shyng and Nichols, 1998). However, as shown in Fig. 3, the R177A mutant subunits can be functionally rescued by coexpression with active Kir6.2 subunits. Kir6.2 [L157C] mutants (Loussouarn et al., 2000) have high intrinsic open state stability, and are relatively insensitive to ATP, with K_{1/2,ATP} ~1 mM (Enkvetchakul et al., 2000). Coexpression of L157C and R177A subunits

²Wild-type channels have a P_{o,zero} of ~0.45 under normal conditions, and this rises to a maximum of ~0.9 after addition of PIP₂, so the macroscopic relative current approximately doubles. Mutations that reduce the apparent PIP₂ affinity will reduce the ambient P_{o,zero}, and this means that the potential increase of P_{o,zero} after addition of PIP₂ is greater. However, unless PIP₂ efficacy is reduced, or the affinity is reduced so far that it becomes impossible to add sufficient PIP₂ (as may in fact be the case for R206A), P_{o,zero} would still rise eventually to the same saturating value (~0.9). Hence, the increase in the relative current will be greater, but take longer, than in wild-type channels.

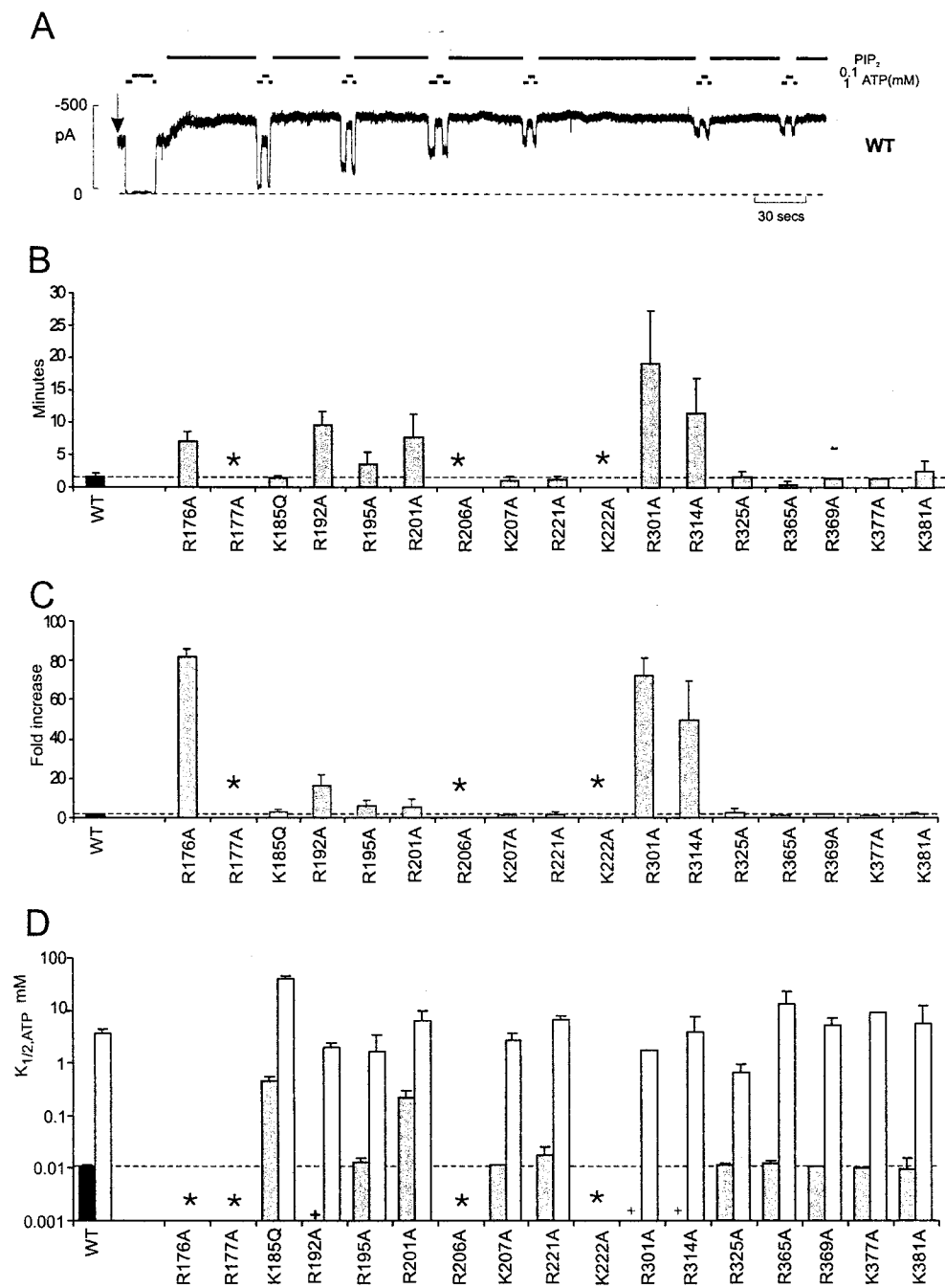


FIGURE 2. Mutation of certain basic residues affects channel response to PIP₂. (A) Representative current recorded from inside-out membrane patch containing wild-type Kir6.2 + SUR1 subunits (WT). In this and subsequent figures, the patch was excised at the arrow, and the bars indicate the application of PIP₂ (5 μg/ml, unless indicated) or ATP (as shown). The dashed line indicates zero current. Inward currents are shown as upward deflections in this and subsequent figures. (B) Time taken for 95% response to PIP₂ for mutant Kir6.2 + SUR1 channels. (C) Fold increase in steady state patch current after addition of PIP₂ (mean ± SEM, *n* = 3–9 in each case). (D) Mean $K_{1/2,ATP}$ for mutant Kir6.2 + SUR1 channels after patch excision (shaded) and after treatment with PIP₂ (*n* = 2–7 in each case, mean ± SEM for mean of 3 or more). Asterisks in B–D indicate mutations for which there was no channel activity in excised patches; plus signs indicate where $K_{1/2,ATP}$ could not be measured reliably on inactivating mutants in D. Dashed lines in B–D indicate wild-type mean.

with SUR1 generated channels that were much more sensitive to ATP (Fig. 3 B; mean $K_{1/2,ATP}$ = 0.18 mM) than L157C + SUR1 alone. This rescue of channel activity by coexpression confirms that the R177A mutation results in channels that are closed, which is consistent with reduced PIP₂ affinity, and not in any gross structural defect.

Nonidentical Residues Control ATP and PIP₂ Sensitivity

All expressed mutants had comparable intrinsic ATP sensitivity to wild-type channels (Fig. 2 D) except K185Q

and R201A. After PIP₂ stimulation, $K_{1/2,ATP}$ increased to between 1 and 10 mM for all mutants. Of the PIP₂-sensitive residues, only R201A (Fig. 4) also affects sensitivity to ATP. Compared with wild-type channels, R201A mutant channels actually showed only moderately increased stimulation by PIP₂ (Fig. 2) and no significant reduction of ⁸⁶Rb efflux in intact cells (Fig. 1 B). However, R201A channels showed a significantly decreased ATP sensitivity (K_i ~115 μM; Fig. 4 B). Since the mutation reduces ATP sensitivity without increasing $P_{o,zero}$, R201 is a candidate ATP binding site residue. A change in ATP binding affinity might also result in altered co-

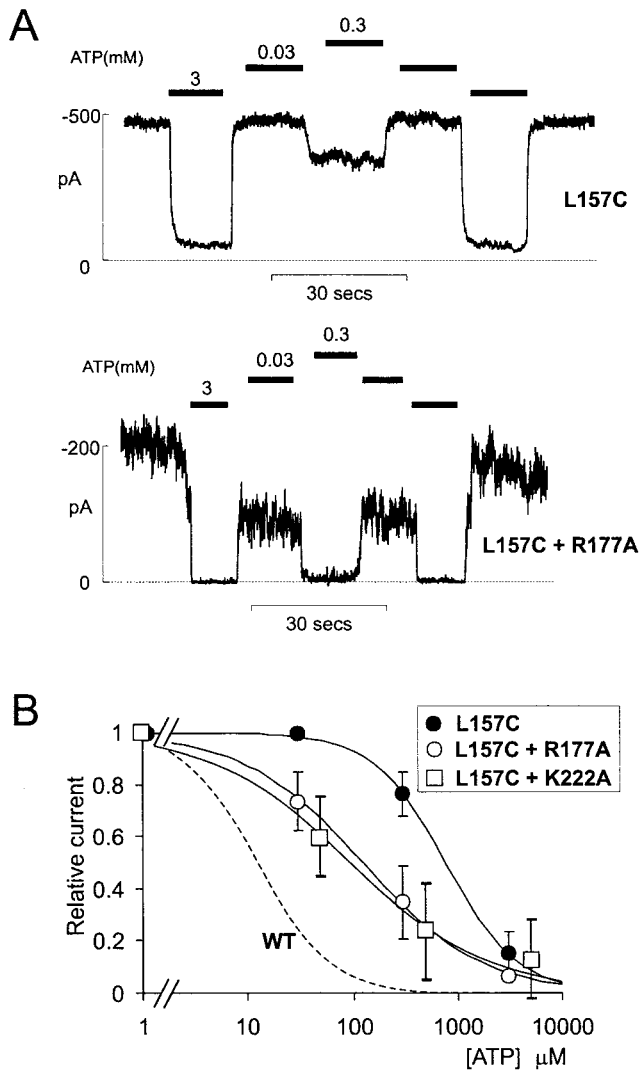


FIGURE 3. Inactive mutants can be rescued by coexpression. (A) Representative currents recorded from an inside-out membrane patch containing Kir6.2[L157C] + SUR1 channels (L157C, above) and from a patch containing mutant Kir6.2[L157C] and Kir6.2[R177A] subunits coexpressed with SUR1 (L157C + R177A, below). (B) Steady state dependence of membrane current on [ATP] (relative to current in zero ATP) for patches as in A and for coexpressed Kir6.2[L157C] and Kir6.2[K222A] subunits, with SUR1 (mean \pm SEM, $n = 5-7$). The data were fit using a Hill equation (relative current = $1 / \{1 + ([ATP]/K_{1/2})^H\}$), with $K_{1/2,ATP} = [ATP]$ causing half-maximal inhibition and H free to vary. Dashed line indicates relationship for wild-type Kir6.2 + SUR1 (WT).

operativity between subunits, which could be an explanation for the experimentally significant reduction of the Hill coefficient (H) for inhibition by ATP (Fig. 4 B). The shift of ATP sensitivity in this mutation is similar to that observed for the K185Q mutant (Fig. 2 D), which has lower affinity for ATP binding (Tanabe et al., 1999; Tucker et al., 1998), but wild-type open state stability and PIP₂ response (Fig. 2).

Like R177A, the R206A and K222A mutant channels

displayed no activity after membrane excision. There was no detectable response of either mutant to a 10-min exposure to 5 μ g/ml PIP₂ (not shown), but R206A channel activity did appear after several minutes of exposure to a much higher concentration of PIP₂ (100 μ g/ml; Fig. 4 C), indicating that the lack of current after patch excision reflects a much lower open state stability. The K222A mutant showed no activity even on exposure to 100 μ g/ml PIP₂. However, like the R177A mutant, K222A channels could be rescued by coexpression with L157C mutants (Fig. 3 B), generating channels that were much more sensitive to ATP (Fig. 3 B; $K_{1/2,ATP} = 0.085$ mM) than L157C + SUR1 alone. Again, this result indicates that the lack of current in the K222A mutant results from channels being closed, which is consistent with reduced PIP₂ affinity, and not from any gross structural defect.

Some Mutants Display Prominent PIP₂-sensitive Inactivation

Interestingly, three of the six mutations (R192A, R301A, and R314A) showed inactivation after removal of ATP, and this inactivation was most pronounced in the R301A mutant (Fig. 5). After patch excision, the estimated time constant of inactivation, after a step from 1 mM ATP to zero ATP, was 10.9 ± 0.5 s for R192A, 2.7 ± 0.3 s for R301A, and 19.5 ± 4.7 s for R314A ($n = 4-10$ patches). We previously observed similar inactivation in an M2 pore mutation (N160Q; Shyng et al., 1997a). The underlying mechanism of such ATP-dependent inactivation remains unclear, but may represent a time-dependent change in open state stability after ATP removal (i.e., a transient high stability of the open state, followed by a subsequent conformation rearrangement that leads to a reduction of open state stability and a low steady state open probability). In every case, after application of PIP₂, this inactivation disappears (Fig. 5), as open state stability increases and ATP sensitivity decreases. Interestingly, the ⁸⁶Rb efflux from mutants that showed inactivation in ATP-free solutions correlated with the severity of the inactivation. Efflux through the very rapidly inactivating R301A mutant was almost background, whereas efflux through R192A and R314A mutants was ~ 85 and $\sim 70\%$ of wild-type, respectively (Fig. 1 B). Given the stimulatory effect of MgADP in intact cells, and that PIP₂ reverses the inactivation process, it is conceivable that these agents sustain activity of the less severe inactivation mutants (R192A and R314A) in intact cells.

DISCUSSION

Distinct Residues Are Involved in Channel Gating of Kir6.2 Channels by ATP and PIP₂

The mechanism of PIP₂ activation of inward rectifier channels, and of ATP inhibition of K_{ATP} channels, re-

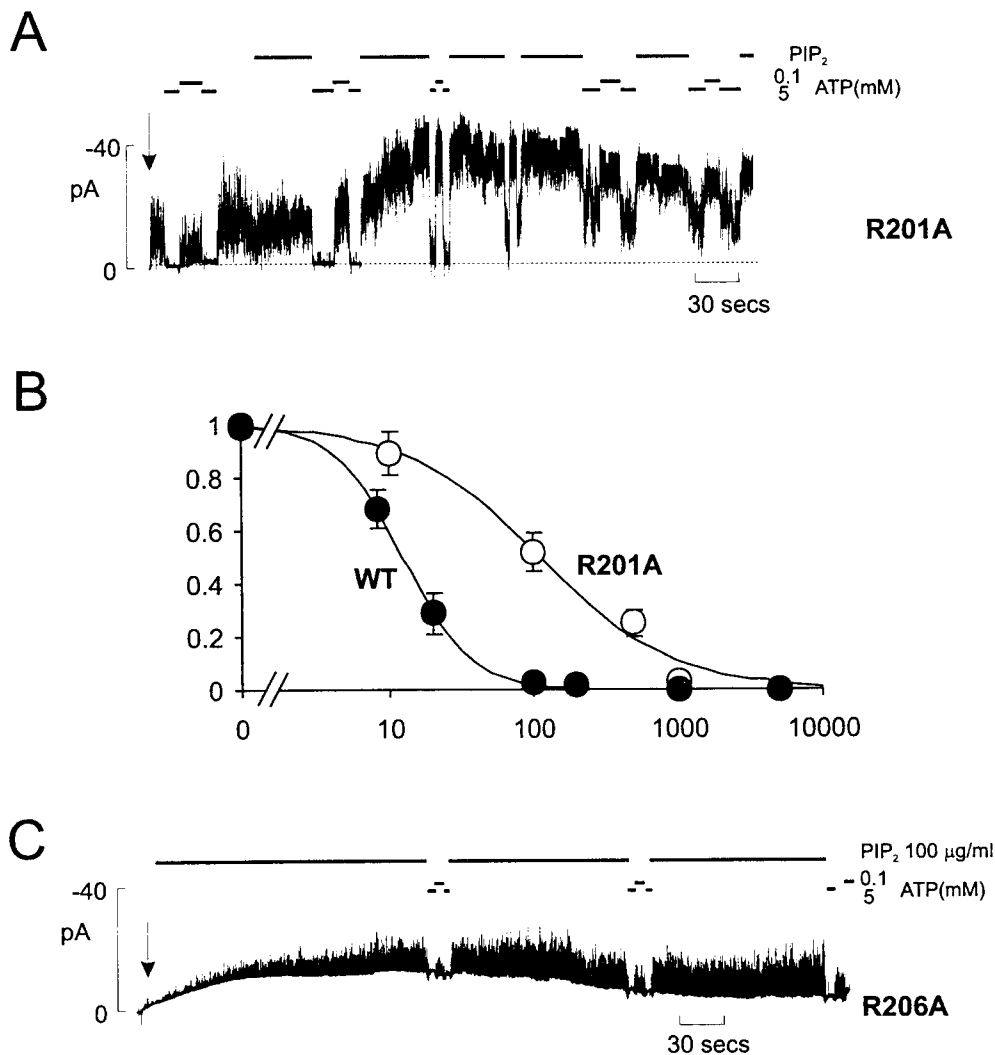


FIGURE 4. R201A mutants alter sensitivity to PIP₂ and ATP. (A and C) Representative currents recorded from inside-out membrane patch containing Kir6.2[R201A] and Kir6.2[R206A] subunits coexpressed with SUR1 subunits. (B) Steady state dependence of membrane current on [ATP] (relative to current in zero ATP) for Kir6.2[R201A] or wild-type Kir6.2 subunits coexpressed with SUR1 (mean \pm SEM, $n = 5-7$). The data were fitted using the Hill equation, with $K_{1/2,ATP} = 12 \mu\text{M}$ (WT) and $115 \mu\text{M}$ (R201A), and $H = 1.9$ (WT) and 0.9 (R201A).

main elusive. It has been suggested previously that electrostatic interaction of the Kir subunit cytoplasmic domain with phospholipids in the membrane stabilizes the open state of the channel (Fan and Makielski, 1997). We propose that ATP and PIP₂ are negative heterotropic regulators of the Kir6.2 channels, such that binding of ATP at a nonidentical site stabilizes the closed channel (Shyng and Nichols, 1998; Enkvetchakul et al., 2000). No high resolution structures are available for the cytoplasmic domains of Kir channels, and there is no homology of these domains to any known structures. Extensive mutagenesis of the Kir6.2 subunit reveals very few residues that are likely to be involved in binding ATP (Drain et al., 1998; Tucker et al., 1997; Enkvetchakul et al., 2000). In the NH₂ terminus of Kir6.2, R50 is one such residue (Tucker et al., 1998) and, in the COOH terminus of Kir6.2, one particularly basic residue (K185) has been shown to affect ATP binding (Tucker et al., 1997; Koster et al., 1999; Tanabe et al., 1999). Neutralization of this residue (K185Q) had no effect on channel response to PIP₂ (Fig. 2). Mu-

tagenesis of Kir1.1, 2.1, and 3.1 channels highlights specific regions of the Kir COOH termini that are significant determinants of PIP₂ sensitivity (Huang et al., 1998; Zhang et al., 1999), and the present study indicates that distinct sets of basic residues regulate the channel response to ATP and PIP₂. Seven mutations caused a significant reduction of channel activity in intact cells. All of these, plus two additional mutations, were shown to reduce the sensitivity to activation by PIP₂ in excised membrane patches. In every case, even when no channel activity was present in excised patches (R177A, R206A, and K222A), channel activity could be detected in coexpression or after prolonged PIP₂ treatment. Thus, none of the mutations caused global structural changes that made subunits nonfunctional, instead the results are consistent with lower open state stability due to reduced PIP₂ sensitivity. Only the R201A mutation affected both ATP and PIP₂ interaction, reducing the sensitivity to both ligands (Fig. 2), which is consistent with the idea that each ligand interacts with separate, but possibly overlapping, sites on the same

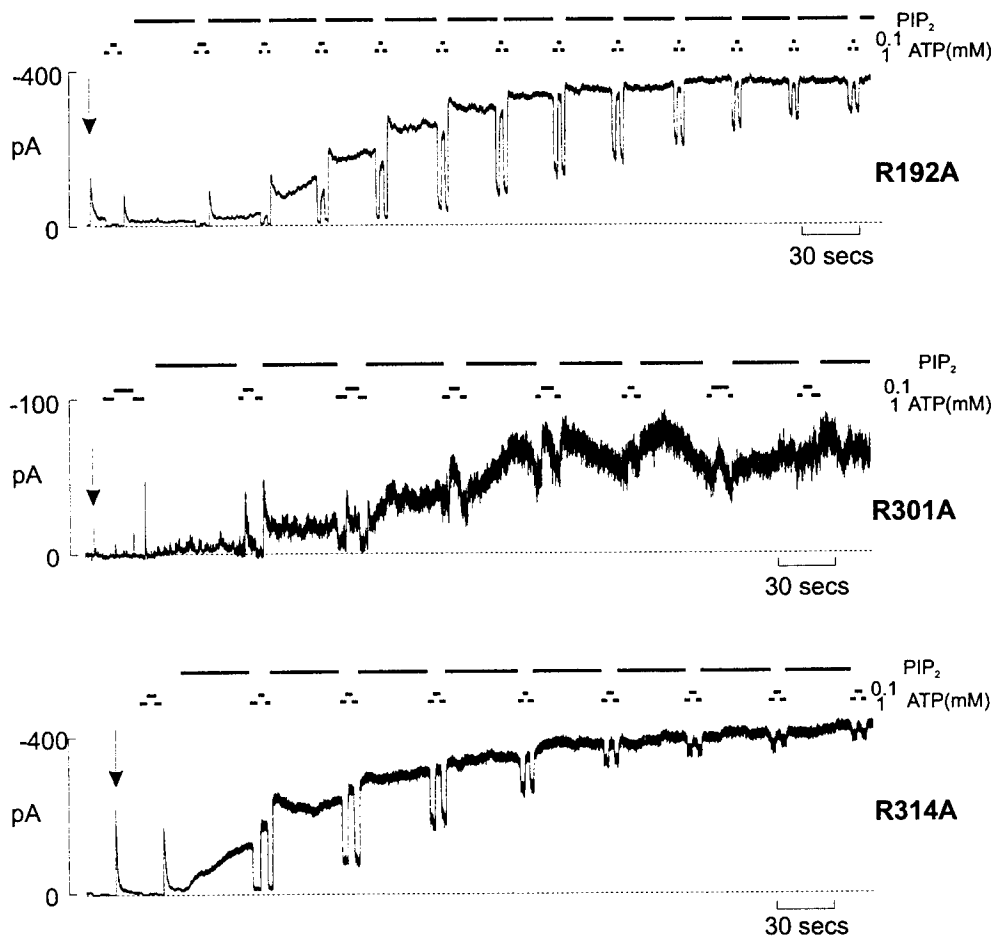


FIGURE 5. Inactivation is induced by some neutralization mutations. Representative currents recorded from inside-out membrane patch containing R192A, R301A, and R314A mutant Kir6.2 subunits coexpressed with SUR1 subunits (see Fig. 2 legend). Note that PIP₂ treatment abolishes inactivation.

domain to stabilize either the closed (ATP) or open (PIP₂) states.

Regulation of Other Inwardly Rectifying Potassium Channels by Membrane PIP₂

Other inward rectifier channels in the Kir family are regulated by membrane PIP₂ (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Huang et al., 1998; Zhang et al., 1999). Using a PIP₂ antibody sensitivity assay, Huang et al. (1998) showed that neutralization of R188 in Kir1.1 (equivalent to R177 in Kir6.2), enhanced sensitivity of channel activity to PIP₂ antibodies and reduced PIP₂ binding to the purified Kir1.1 COOH terminus. Several other residues contribute to differential interaction of PIP₂ with Kir3.4 (GIRK4) and Kir2.1 (IRK1), including the K207-L245 region of Kir2.1 (corresponding to R196-L233 in Kir6.2). When this segment of Kir2.1 is introduced in place of the corresponding segment into Kir3.4, the resultant chimera shows Kir2.1-like sensitivity to PIP₂ antibodies (Zhang et al., 1999).

The regions equivalent to residues 206–222 in Kir6.2 have been mutated in several studies on various Kir

channels. E224 in Kir2.1 (S212 in Kir6.2) is a major determinant of the affinity for pore-blocking polyamines (Yang et al., 1995). The neighboring residue (H216) in Kir6.2 also controls pH dependence of polyamine block (Baukrowitz et al., 1999), and a recent cysteine scanning mutagenesis of this region in Kir2.1 (Lu et al., 1999) indicates that several residues within it are accessible to methyl-thio-sulphydryl reagents. Together, these results are consistent with this region being accessible to permeant ions and forming part of the cytoplasmic entrance to the pore. However, many mutations in the pore-lining M2 region also cause changes in P_{o,zero} (Loussouarn et al., 2000) and alter the responsiveness to PIP₂ (Enkvetchakul et al., 2000). Since these residues are clearly lining the pore within the membrane itself, they presumably cannot interact with phospholipid headgroups. In the latter case, we can reasonably conclude that the effect of the mutation is on intrinsic pore stability (i.e., transduction of PIP₂ action) rather than on PIP₂ binding. Based on the above evidence that the 206–222 region is likely also to be pore-lining, and therefore, unlikely to be interacting directly with membrane PIP₂ headgroups, we speculate that the effects of mutations (e.g., R206A and R222A) in this region on

PIP₂ sensitivity also result from alterations in open pore stability rather than on PIP₂ binding.

Critical residues involved in PIP₂ regulation are conserved, or appropriate changes in PIP₂ sensitivity are observed, when such residues are introduced to different Kir channels (Huang et al. 1998; Zhang et al., 1999). Although the physiological behaviors of Kir channel family members appear quite different (e.g., strong inward rectifiers in the Kir2 subfamily, G-protein-gated channels in the Kir3 family, and K_{ATP} channels in the Kir6 family), there is a growing convergence of their fundamental molecular nature. All contain essentially similar pore structures, and strong versus weak rectification can be conferred or removed by one or two point mutations within the pore. All are activated by PIP₂, and homologous residues control open state stability and PIP₂ sensitivity. One question that arises is: Why are only Kir6 subfamily members ATP-sensitive? Recent studies demonstrate that the relatively high ATP sensitivity of Kir6.2 channels is labile; point mutations can render the channel very ATP-insensitive (Tucker et al., 1997, 1998; Drain et al., 1998; Trapp et al., 1998; Enkvetchakul et al., 2000; Loussouarn et al., 2000), and any mutation, or manipulation, that increases open state stability reduces the apparent ATP sensitivity. Accordingly, while the lack of ATP sensitivity in other inward rectifiers might be due to the lack of an appropriate binding site, it may be simply a consequence of altered pore stability. Interestingly, there have been reports of weak ATP sensitivity of several Kir subfamily members, including Kir2.3 (Collins et al., 1996), Kir1.1 (McNicholas et al., 1996), and Kir4.1 (Bredt et al., 1995).

The present study was undertaken without any knowledge, or presupposition, of the overall structure of the cytoplasmic domain. In the absence of any homology to known structures, we performed a prediction of the secondary structure of the COOH terminus of Kir channels using multiple alignments of the primary sequences (PHD program [Rost, 1996]). This prediction indicates that the first ~150 amino acids of the COOH termini of all Kir channels are likely to contain seven antiparallel β -strands with an α -helix at the COOH-terminal end. These are characteristics of pleckstrin homology (PH) domains (Lemmon et al., 1996; Shaw, 1996; Rebecchi and Scarlata, 1998), which are found in many PIP₂-interacting proteins. In such proteins, multiple positively charged residues in the loops between the β -strands are responsible for the interaction with PIP₂. Although present in a large number of signaling molecules, PH domains are elusive and difficult to recognize on the basis of sequence homology, and have not been reported in any ion channels. A high resolution structure of Kir channel cytoplasmic domains may well soon appear. However, until such a

time, although mere speculation, the possibility that the PIP₂ sensitivity of inward rectifiers arises from a PH-like domain should perhaps be considered.

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