

Structural and Functional Determinants of Conserved Lipid Interaction Domains of Inward Rectifying Kir6.2 Channels

CATHERINE A. CUKRAS, IANA JELIAZKOVA, and COLIN G. NICHOLS

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110

ABSTRACT All members of the inward rectifier K^+ (Kir) channel family are activated by phosphoinositides and other amphiphilic lipids. To further elucidate the mechanistic basis, we examined the membrane association of Kir6.2 fragments of K_{ATP} channels, and the effects of site-directed mutations of these fragments and full-length Kir6.2 on membrane association and K_{ATP} channel activity, respectively. GFP-tagged Kir6.2 COOH terminus and GFP-tagged pleckstrin homology domain from phospholipase C $\delta 1$ both associate with isolated membranes, and association of each is specifically reduced by muscarinic m1 receptor-mediated phospholipid depletion. Kir COOH termini are predicted to contain multiple β -strands and a conserved α -helix (residues ~ 306 – 311 in Kir6.2). Systematic mutagenesis of D307-F315 reveals a critical role of E308, I309, W311 and F315, consistent with residues lying on one side of a α -helix. Together with systematic mutation of conserved charges, the results define critical determinants of a conserved domain that underlies phospholipid interaction in Kir channels.

KEY WORDS: K^+ current • K_{ATP} • PIP_2 • Kir6.2 • PH domain

INTRODUCTION

The opening and closing of ion channel pores is controlled by conformational changes in other parts of the protein, in response to changes in external stimuli. Activities of inward rectifier K^+ (Kir) channels are modulated by diverse second messengers, including ATP (Kir6 sub-family) (Inagaki et al., 1995), pH (Kir1, Kir4 sub-family) (Fakler et al., 1996; Pearson et al., 1999; Yang and Jiang, 1999), and G-proteins (Kir3 sub-family) (Reuveny et al., 1994; Huang et al., 1995), but common to all is control of activity by polyphosphoinositides (PPIs),* particularly phosphatidylinositol-4,5-bisphosphate (PIP_2) (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Rohacs et al., 1999; Zhang et al., 1999; Hilgemann et al., 2001). In essence, the “physiological” second messengers all act by augmentation of, or competition with, the activating effects of PPI, and when these are depleted from the membrane, Kir channel activity disappears (Baukrowitz et al., 1998a; Huang et al., 1998; Shyng and Nichols, 1998; Liou et al., 1999; Zhang et al., 1999). Evidence is accumulating to suggest that PPI, in particular PIP_2 , interact directly with the conserved cytoplasmic regions of the channel and that associated conformational changes are allosterically coupled to pore opening (Baukrowitz et al., 1998a; Huang

et al., 1998; Shyng and Nichols, 1998; Rohacs et al., 1999; Enkvetchakul et al., 2000; Soom et al., 2001). Using site-specific mutagenesis, several sites, particularly within the COOH termini of Kir6.2, have been shown to be involved in activation by PPI (Baukrowitz et al., 1998a; Huang et al., 1998; Shyng and Nichols, 1998; Rohacs et al., 1999; Zhang et al., 1999; Shyng et al., 2000).

All cation channel superfamily members consist of a tetrameric arrangement of homomeric or similar domains, which may be in individual subunits, or covalently linked. Kir channels are composed of four subunits, each containing two transmembrane segments (M1, M2), a P-loop between M1 and M2, and cytoplasmic NH_2 and COOH termini (Nichols and Lopatin, 1997; Doyle et al., 1998). The M2 helices line the inner vestibule of the channel and it has been proposed that this region behaves as a gate, governing the flow of ions through the channel and across the membrane (Liu et al., 1997; Perozo et al., 1999; Loussouarn et al., 2001). Approximately 40 amino acids preceding M1, and 150 amino acids after M2 are highly conserved in Kir channels, but the extreme NH_2 and COOH termini are not. There is currently no secondary or tertiary structural information regarding Kir channels, so despite the detailed picture that is emerging of the pore itself (Doyle et al., 1998), and potential mechanisms of pore opening, the structural basis for the control of gating by intracellular domains is unknown. Using a combination of electrophysiological and biochemical approaches, we show that a conserved cytoplasmic region of Kir channels interacts with cell membranes, and propose that Kir channels contain a unique lipid interaction

Address all correspondence to Colin G. Nichols, Department of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. Fax: (314) 362-7463; E-mail: cnichols@cellbio.wustl.edu

*Abbreviations used in this paper: ACh, acetylcholine; KIRLI, Kir lipid interacting; PIP_2 , phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PPI, polyphosphoinositide; PS, phosphatidylserine.

(KIRLI) domain that may conserve structurally significant features of phospholipid interacting PH domains.

MATERIALS AND METHODS

Molecular Biology

Point mutations were prepared by overlap extension at the junctions of the relevant residues by sequential PCR. Resulting PCR products were subcloned into pCMV6b vector. Before transfection, constructs were sequenced to verify the mutations.

Expression in COSm6 Cells

COSm6 cells were plated at a density of $\sim 2.5 \times 10^5$ cells/well (30 mm six-well dishes) and cultured in Dulbecco's modified Eagle medium plus 10 mM glucose (DMEM-HG), supplemented with FCS (10%). The following day, cells were transfected by adding FUGENE (Roche Applied Science) and 1 μ g each of pCMV6b-Kir6.2 or mutant isoforms, pECE-SUR1 cDNA, and pECE-green fluorescent protein (GFP) directly to the media. The cells were split the next day onto coverslips for patching. For cell fractionation experiments, cells were plated on 60-mm diameter dishes. Transfection was performed using Lipofectamine (Invitrogen) and 12 μ g of GFP fusion protein DNA per dish in DMEM-HG. The next day the DMEM-HG was replaced with complete media. All experiments were performed 36 h posttransfection.

Hypotonic Lysis and Fractionation

Transfected cells were grown to confluence in 60-mm dishes. The cells were washed twice with PBS (37°C) and scraped into PBS (4°C), pelleted by centrifugation, and resuspended in 1 ml hypotonic solution (5 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA) containing mini COMPLETE protease inhibitors (Boehringer Mannheim). The cells swelled on ice for 45 min and were then passaged 60 times through a ball bearing homogenizer and centrifuged for 10 min at 1,000 *g* to remove nuclear debris and unbroken cells. The postnuclear lysate was centrifuged at 100,000 *g* (55,000 rpm in a TLA-100 rotor; Beckman) for 1 h. The pellet was resuspended in an equal volume of hypotonic solution with protease inhibitors and passed through a 21-gauge needle six times for resuspension. For cells treated with acetylcholine (ACh) in Fig. 5, 100 μ M ACh was applied 15 min before swelling and lysis, and ACh was present throughout the remainder of the fractionation.

Fluorescence Measurements

Each 1 ml fraction of either supernatant or pellet was excited at 467 nm in a fluorometer. Emission spectra (480–600 nm) of buffer alone and of untransfected cells were collected for each experiment. GFP fluorescence spectra were obtained by subtraction of the appropriate spectrum from untransfected samples and normalized to protein density estimated from E₄₈₀, i.e., adjusted spectra = (raw spectra – buffer spectra) – constant \times (control spectra – buffer spectra). The fractional fluorescence in the membrane (E[memb]/E[memb + Cyt]), reported in Figs. 4 and 5, was calculated from the emission at 518 nm, with excitation at 467 nm.

Confocal Microscopy

Cells were cultivated for 24–72 h after transfection and photographed in UV light under 1,000 \times magnification in a ZEISS microscope equipped with a 515-nm emission filter. Confocal analysis was performed using an Argon-Krypton laser (Bio-Rad Labo-

ratories). Green fluorescence was detected at $\lambda = 515$ nm after excitation at $\lambda = 488$ nm. Cells were observed on thin coverslips, and digitized confocal images were prepared for presentation using Corel Photopaint (Corel Inc.).

Patch-clamp Measurements

Patch-clamp experiments were made at room temperature, in a chamber which 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 Instruments, Inc.). Standard bath and pipette solutions (K-INT) had the following composition: 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.3. PIP₂ was diluted in K-INT and 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. Data were filtered at 0.5–3 kHz, digitized at 22 kHz (Neurocorder; Neurodata) and stored on videotape. Experiments were replayed onto a chart recorder, or digitized into a computer using Axotape software (Axon Instruments, Inc.) and analyzed off-line using Microsoft Excel. Wherever possible, data are presented as mean \pm SEM. Microsoft Solver was used to fit data by a least-square algorithm.

Secondary Structure Predictions

Secondary structure predictions were made for Kir6.2 using multiple sequence alignments, submitted through the Washington University Structural Genomics web site: <http://www.biochem.wustl.edu/~StrucGen/>.

RESULTS

The Kir6.2 COOH Terminus Associates with the Membrane

PIP₂-interacting PH domains from various proteins have been tagged with GFP (Wang et al., 1996; Nagel et al., 1998; Varnai et al., 1999; Hodgkin et al., 2000) to study their interaction with the membrane. We took advantage of this approach to examine membrane association of isolated fragments of the Kir6.2 subunit of the K_{ATP} channel expressed in COSm6 cells. GFP-tagged constructs, transiently expressed in transfected cells, were analyzed by confocal microscopy (Fig. 1 A) and in a parallel cell fractionation assay (Fig. 1 B). The full-length channel Kir6.2-GFP is visible in the plasma membrane and punctate intracellular structures in intact cells (Fig. 1 A), and is >90% associated with the isolated membrane fraction (Fig. 1 B). In contrast, GFP alone shows a cytoplasmic distribution (Fig. 1 A) and is >90% associated with the isolated cytoplasmic fraction (Fig. 1 B), consistent with a soluble protein that does not interact with the membrane. The isolated COOH terminus of Kir6.2 (residues 170–390) tagged with GFP ([170–390]-GFP) also shows plasma membrane and intracellular localization under confocal microscopy (Fig. 1 A). In the fractionation assay, \sim 45% of the fluorescence is associated with the membrane fraction (Fig. 1 B). The PH domain of phospholipase C δ 1 (PLC δ 1) tagged with GFP (GFP-PH) (gift of Tobias Meyer, Stan-

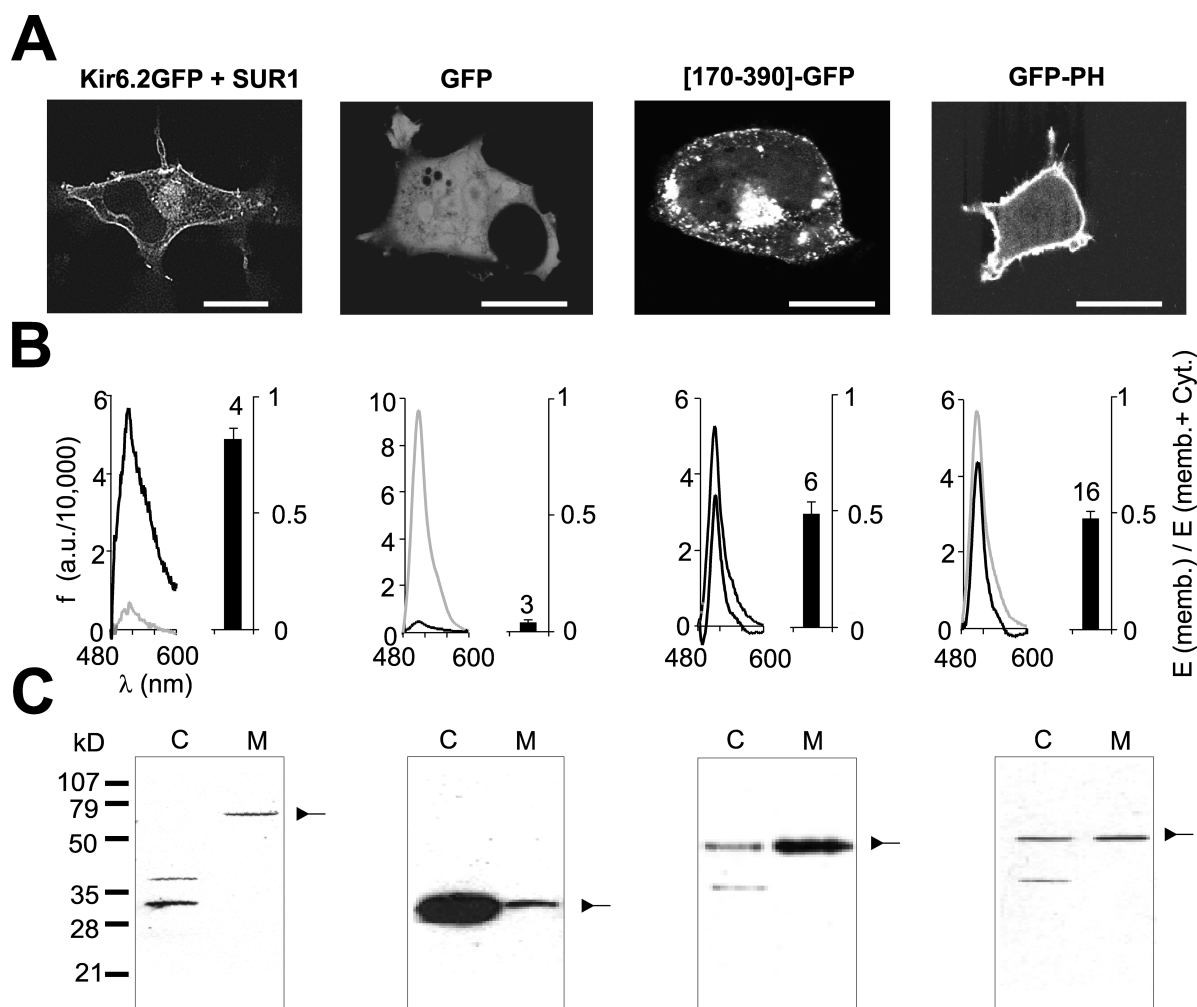


FIGURE 1. Membrane association of Kir6.2 COOH terminus. (A) Confocal fluorescent images of COSm6 cells expressing Kir6.2-GFP+SUR1, GFP, [170-390]-GFP, or GFP-PH. Calibration bars represent 3 μ m. (B, left) Representative emission spectra of membrane (black) and cytosolic (gray) fractions from transfected cells as in A (see MATERIALS AND METHODS). (right) Fraction of fluorescence in membrane (mean \pm SEM) from similar experiments, n indicated. (C) Western blot using polyclonal anti-GFP antibody of cytosolic (C) and membrane (M) fractions as in B. Arrows indicate expected size of tagged constructs. There is a probable contaminant protein at \sim 40 kD in cytosolic fractions. Cytosolic anti-GFP reactivity at \sim 30 kD may represent cleaved GFP in full-length Kir6.2-GFP assay.

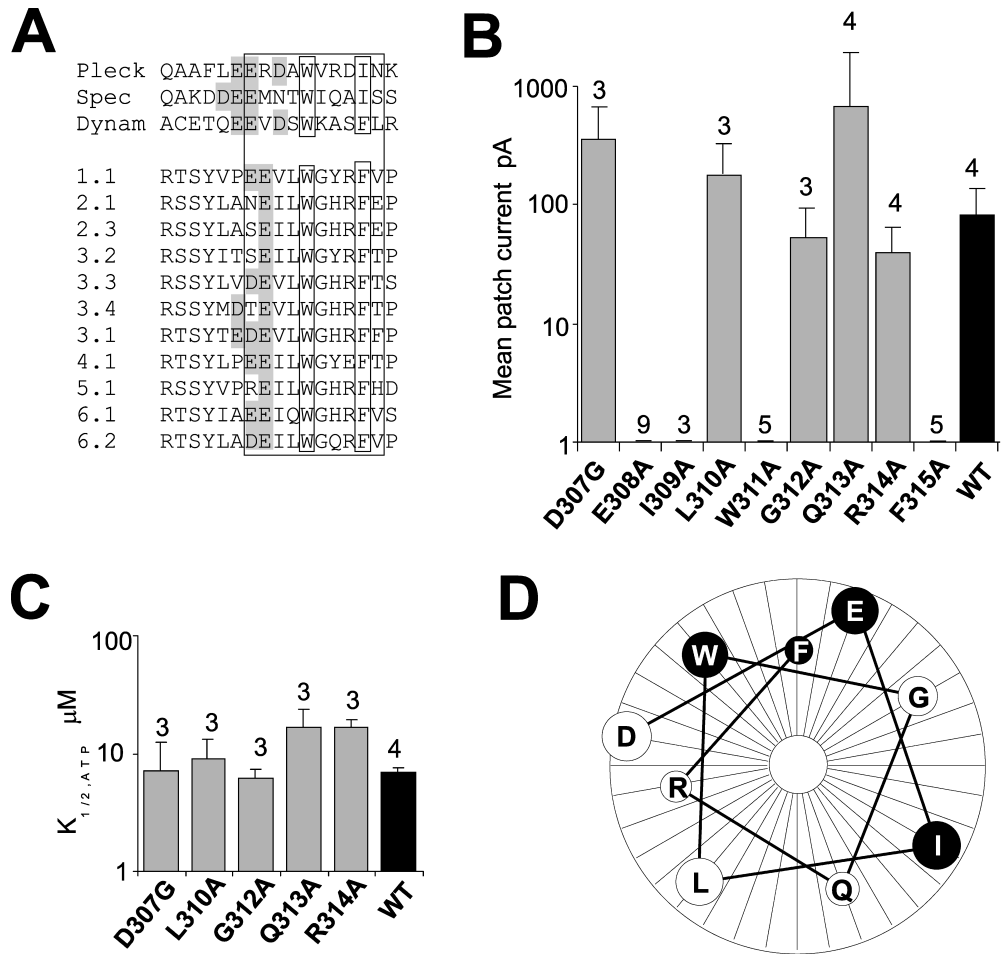
ford University, Stanford, CA) was used as a positive control for a protein known to bind to phospholipids (specifically PI-4,5-bisphosphate) (Cifuentes et al., 1994; Lemmon et al., 1996) in the membrane. Cells transfected with this construct show GFP fluorescence in a thick band outlining the membrane of the cell. In the fractionation assay, the membrane association of the GFP-PH construct was very similar (\sim 45%) to that of [170-390]-GFP. Importantly, as shown in Fig. 1 C, all constructs are expressed as a single protein of appropriate molecular weight.

Alanine Scan of Putative C-helix: Mutations of Conserved Residues Abolish Channel Activity

The cytoplasmic regions of Kir channels are highly conserved in the \sim 40 amino acids preceding the M1 seg-

ment (residues 34-78 in Kir6.2) and \sim 150 amino acids following M2 (residues 170-320 in Kir6.2), although they share no significant homology with any known protein domains. We performed predictions of the secondary structure of Kir channels using alignments of these primary sequences using various prediction algorithms (see Fig. 6 A, and DISCUSSION). The conserved COOH terminus is predicted to contain primarily β -strands and loops but only one significant α -helix (C-helix, approximately residues 306-311). To examine the likely structure and significance of this region, we performed a systematic mutagenesis of residues 307-315, replacing each residue with alanine. Functional channels were not obtained with mutations of residues E308, I309, W311, and F315, but essentially normal channel activity was observed for all other residues be-

FIGURE 2. Conserved α -helix of Kir COOH terminus. (A) Conserved COOH-terminal α -helix of PH domain (boxed) of pleckstrin (Pleck), spectrin (Spec), and dynamin I (Dynam) aligned with predicted COOH-terminal α -helix of inward rectifiers (indicated by subfamily nomenclature). The conserved Trp (W311 in Kir6.2) and large hydrophobic (F315 in Kir6.2 or I) residues are boxed, upstream negative charges are shaded in gray. (B) Mean patch current for D307G and alanine-substituted mutants (E308A-F315A). No currents were recorded from E308A, I309A, W311A, and F315A mutants. (C) $K_{1/2,ATP}$ for mutant channels (mean \pm SEM, n indicated). (D) Helical projection of residues D307–F315. Residues for which channel activity was abolished when mutated are highlighted in black.



tween D307 and R314 (Fig. 2, B and C). On a helical wheel projection (Fig. 2 D), residues E308, I309, W311, and F315 are all on the same face and E308, W311, and F315 are predicted to be separated by single turns of the helix.

In a previous systematic mutagenesis of positive charges in the Kir6.2 COOH terminus (Shyng et al., 2000), it was striking that all 22 mutants could produce functional subunits, although in some cases, channel activity was observed only after application of PIP_2 , or after coexpression with high open state stability mutants such as Kir6.2[L157C], which expresses with high open probability and low ATP sensitivity (Enkvetchakul et al., 2000). Substitution of W311 or F315 with A, R, or D results in no detectable channel activity even after application of 100 μ g/ml PIP_2 ($n = 3-5$ patches in each case; unpublished data). Moreover, W311A and F315A mutants are not rescued by coexpression with L157C mutants (Fig. 3 A). W311A and F315A mutants actually cause a strong dominant-negative suppression of L157C subunits (Fig. 3 A). The only detectable currents correspond to those expected of pure L157C+SUR1 currents (Fig. 3 B), consistent with in-

corporation of even one W311A or F315A subunit into a tetramer being sufficient to abolish activity.

The Conserved Kir6.2 COOH Terminus Interacts with the Membrane

We next attempted to define structural boundaries of the KIRLI domain. The COOH-terminal 36 amino acids of Kir6.2 (residues 354–390) contain an endoplasmic reticular retention signal which affects channel trafficking to, but not function at, the membrane (Tucker et al., 1997; Zerangue et al., 1999; Ma et al., 2001). Fig. 4 A shows that the [170–354]-GFP fragment associates with the membrane slightly less effectively than the full length COOH terminus (i.e., [170–390]-GFP), but no further reduction of association is seen when the COOH terminus is reduced to the highly conserved (i.e., [170–320]-GFP) fragment (Fig. 4 A). However, truncation of a further 20 amino acids (i.e., [170–300]-GFP), which removes the putative C-helix, abolishes membrane association (Fig. 4 B). Deletions into the conserved domain from the NH_2 terminus also considerably reduce association with the membrane and the short [293–320]-GFP has a similar cellular distribu-

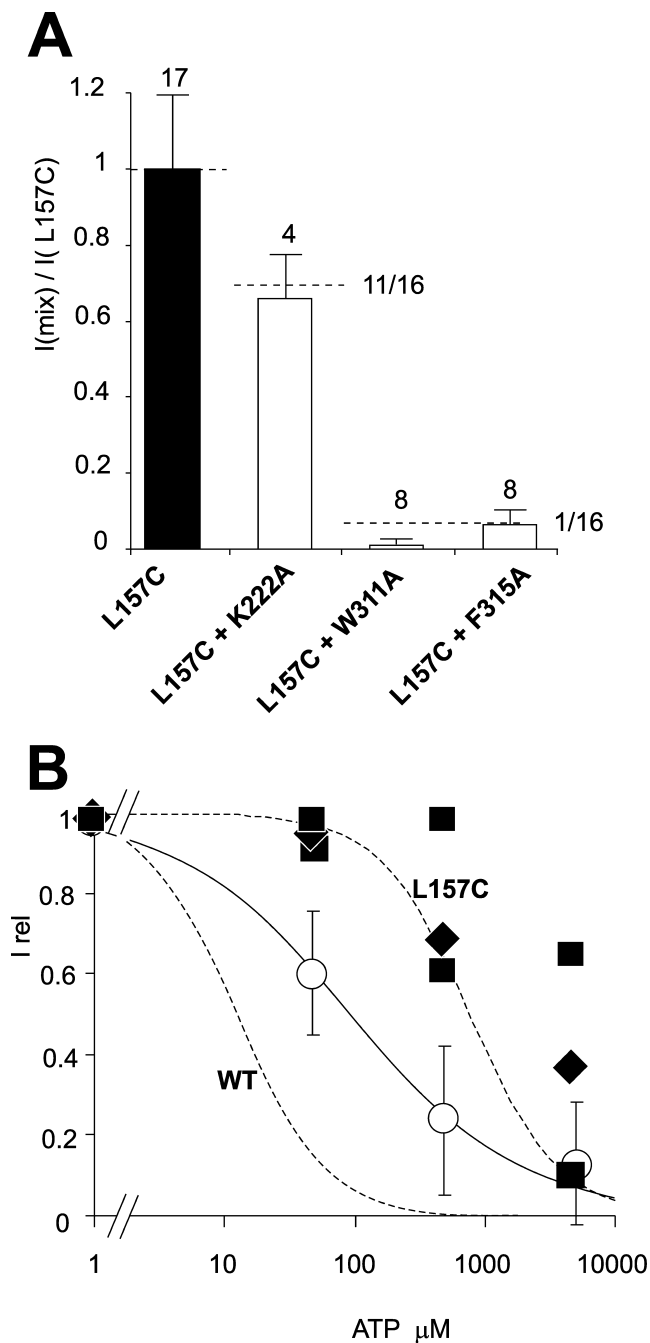


FIGURE 3. Dominant-negative inhibition by W311A and F315A mutants. (A) Current density (in zero ATP) in patches expressing Kir6.2[L157C]+SUR1 (L157C), or Kir6.2[L157C]+SUR1 coexpressed with other mutant Kir6.2 subunits, relative to currents in Kir6.2[L157C]+SUR1 patches. Dashed lines indicate predictions assuming that subunits are expressed at equal density, and that at least three K222A subunits are necessary to inhibit current, or only one W311A or F315A subunit is sufficient to inhibit activity. (B) Steady-state dependence of membrane current on [ATP] (relative to current in zero ATP, I_{rel}) for wild type or L157C mutant channels (dashed, from [Shyng et al., 2000]). Superimposed data points are from individual patches in which L157C subunits were coexpressed with K222A (○), W311A (■), or F315A (◆) mutant subunits.

tion to GFP alone (Fig. 4 B). Thus, we propose that residues 170–320 comprise the core of the KIRLI domain. To examine the role of W311 and F315 in membrane association, we introduced W311A and F315A mutants in the [170–320]-GFP construct. Each of these mutations affected expression level. In parallel transfections, fluorescence of W311A and F315A mutations were $28 \pm 15\%$ ($n = 4$) and $4 \pm 3\%$ ($n = 3$) of that of the wild type construct, respectively, suggesting instability of the expressed proteins. Only the fluorescence of the W311A mutant was high enough to quantify membrane association in the fractionation assay. As shown in Fig. 4 C, this point mutation significantly reduced the ability of this fragment to associate with the membrane.

m1 Receptor Activation Reduces Membrane Association of both the Kir6.2 KIRLI Domain and the PLCδ1 PH Domain

It has been demonstrated previously that PLC activation via m1 receptor stimulation causes a decrease of K_{ATP} current, consistent with reduced channel- PIP_2 interactions (Xie et al., 1999). Other studies have dynamically monitored membrane PIP_2 itself, making use of PLCδ1 PH domain tagged with GFP (GFP-PH) (Stauffer et al., 1998; Varnai and Balla, 1998) to provide a relative estimate of PIP_2 and IP_3 in response to m1 receptor stimulation and resulting PIP_2 hydrolysis. Kobrinsky et al. (2000) used this approach to demonstrate redistribution of GFP-PH in transfected cells using confocal imaging. We have used the same principle to develop a quantifiable assay for dynamic redistribution of GFP-tagged channel fragments (Fig. 5). To quantify membrane association, cells were fractionated either in the presence or absence of ACh (see MATERIALS AND METHODS). Fig. 5 A quantifies membrane association of GFP-PH expressed alone, and with muscarinic m1 or m2 receptors, from cells incubated in the presence or absence of 100 μ M ACh. The membrane association of GFP-PH is specifically reduced only when the m1 receptor is expressed and activated by ACh. Similarly, [170–320]-GFP fluorescence is only reduced by m1 receptor activation (Fig. 5 B). In contrast, the distribution of fluorescence of full length Kir6.2-GFP (a transmembrane protein) was unaffected by m1 receptor activation (Fig. 5 B).

DISCUSSION

PIP₂ Interaction with Kir Channels

It is now clear that PPIs, in particular PIP_2 , can have a profound activating effect on all inward rectifier K^+ (Kir) channels (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Baukrowitz et al., 1998b; Zhang et al., 1999; Hilgemann et al., 2001). Actin stabilizers such as phalloidin can inhibit channel rundown while depolymerizing agents such as cytochalasins can accelerate

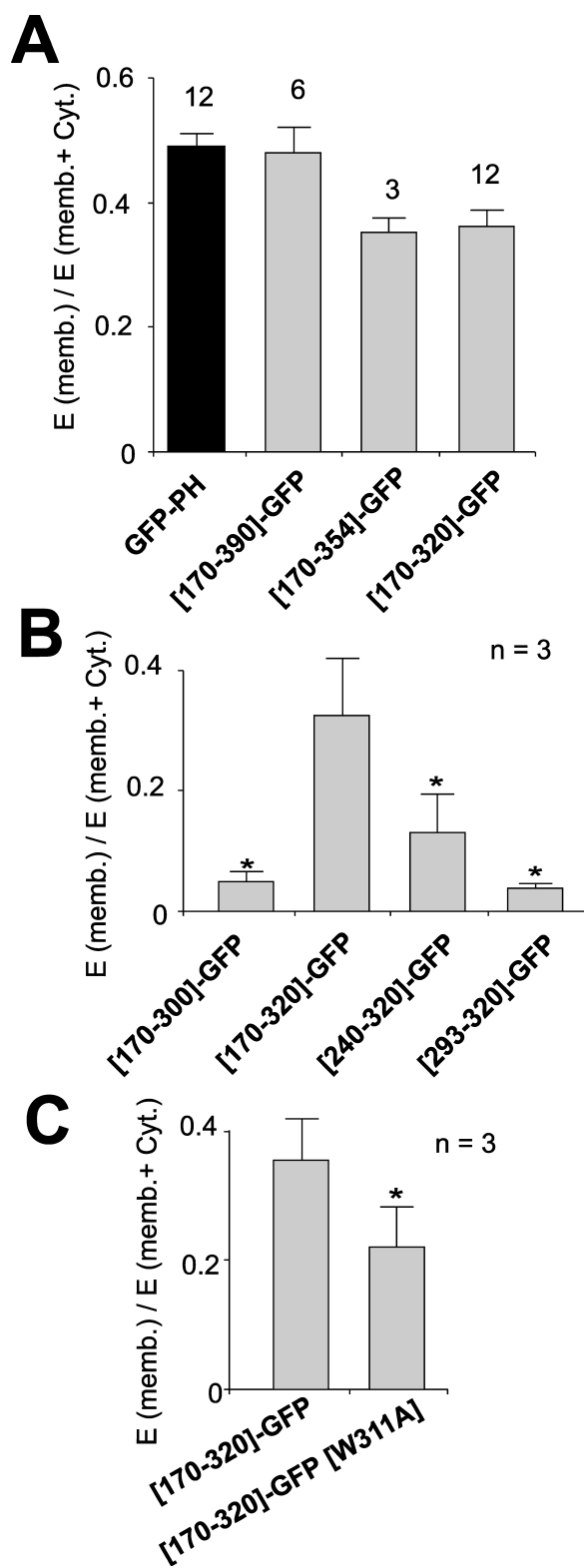


FIGURE 4. Membrane association of Kir6.2 COOH-terminal fragments. (A–C) Fraction of fluorescence in membrane (mean \pm SEM) from cells expressing GFP-PH and GFP-tagged COOH-terminal fragments (mean \pm SEM, *n* indicated). In B and C, results are shown from parallel transfections (*n* indicated, *, $P < 0.05$).

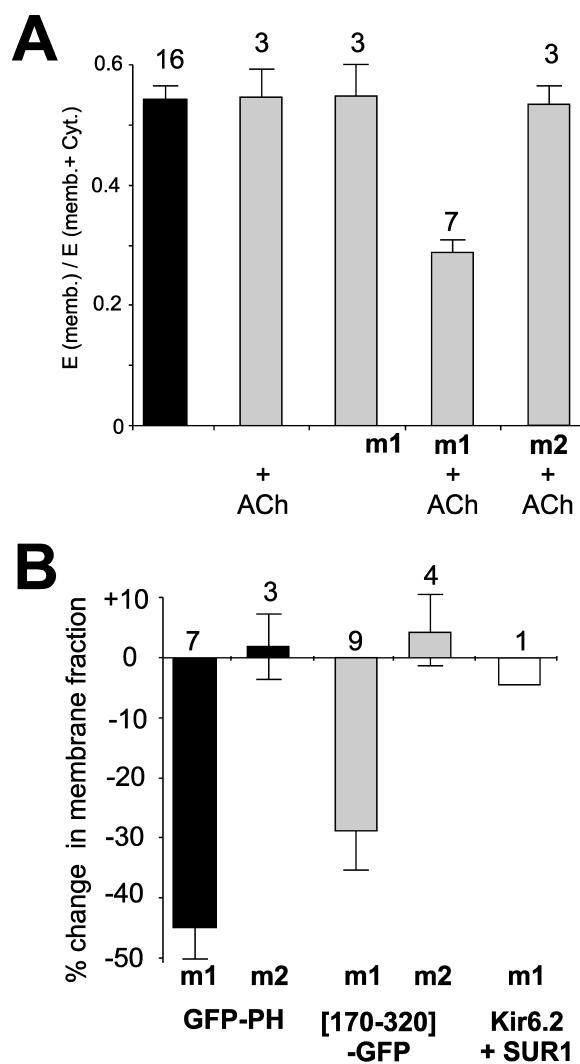


FIGURE 5. Specific decrease of membrane association following M1 receptor activation. (A) Fraction of fluorescence in membrane (mean \pm SEM) from cells expressing GFP-PH and m1 or m2 receptors in the presence or absence of ACh, as indicated. (B) Percent change in fraction of fluorescence in the membrane upon addition of ACh for GFP-PH or channel constructs coexpressed with m1 or m2 receptors, as indicated (mean \pm SEM, *n* indicated).

channel rundown (Furukawa et al., 1996; Harvey et al., 2000), leading to initial suggestions that the action of PIP_2 was via cytoskeletal interactions. This idea has not been ruled out, but the weight of evidence indicates a direct action on Kir subunits, specifically involving interaction with the cytoplasmic COOH termini. Since most cytoskeletal proteins interact with phosphoinositides (PIP_2 or PIP_3) (Janney, 1994), the demonstration that phosphatidylserine (PS) and other negatively charged lipids, such as acyl CoA, can also activate Kir channels, argues against a cytoskeletal intermediate. The most direct evidence is the finding that specific mutations in the COOH terminus can reduce both channel activity in cell membranes and binding of PIP_2

to isolated fragments (Fan and Makielski, 1997; Huang et al., 1998; Shyng and Nichols, 1998; Soom et al., 2001) and the apparent antagonism of ATP inhibition and PPI activation of K_{ATP} channels (Baukrowitz et al., 1998b; Shyng and Nichols, 1998; Enkvetchakul et al., 2000).

A Cytoplasmic Membrane-interacting Domain in Kir Channels

Recent studies with PLC δ 1 PH domain tagged with GFP (GFP-PH) have dramatically demonstrated localization of this domain to the cell membrane. One goal of the present study was to develop a physiological assay of membrane association of isolated Kir6.2 fragments, to test the hypothesis that a discrete domain exists and that it binds to the cell membrane in a PPI-dependent manner. Confocal fluorescence images indicate that GFP-tagged full-length Kir6.2 constructs are localized to the cell membrane, but are also retained in intracellular compartments (Makhina and Nichols, 1998) (Fig. 1 A). A visually similar pattern is observed for the isolated COOH terminus ([170–390]-GFP) that differs from the almost exclusively edge fluorescence observed for the PLC δ 1 PH domain (GFP-PH). Importantly, this particular PH domain, but not others, has been reported to have high affinity and specificity for PI-4,5-P₂ (Lemmon et al., 1995). Kir6.2 clearly interacts with multiple PPI species (Fan and Makielski, 1997), so while it is possible that the discrete intracellular fluorescence of [170–390]-GFP is due to retention within an enclosed compartment, it is also possible that the construct is associated with intracellular PPI to which PH-GFP has relatively low affinity.

The fractionation assay allows quantification of the association of GFP-tagged constructs with the membrane fraction, and hence a semiquantitative assay of the relative affinity of a given construct for binding to the membrane. The data indicate that while the conserved COOH terminus ([170–320]-GFP) associates with the membrane almost as well as the complete COOH terminus (170–390]-GFP) or the PLC δ 1 PH domain (GFP-PH), truncations into this domain from either end (Fig. 4 B) greatly reduce association. Therefore, residues 170–320 form the essential core of the COOH-terminal lipid-interacting domain. Activation of muscarinic m1 receptors by ACh results in G_q α -mediated PLC activation and consequent PIP₂ hydrolysis (Caulfield and Birdsall, 1998). In single COS-1 cells co-expressing GFP-PH and m1 receptors, ACh activation decreased membrane association of GFP-PH by 55% (Kobrinisky et al., 2000), consistent with a relative decrease in the PI-4,5-P₂/IP₃ ratio within the cell (Stauffer et al., 1998; Varnai and Balla, 1998). This shift in GFP-PH fluorescence was not seen with activation of co-expressed m2 receptors, which couple to alternative

G-protein-mediated pathways. We have now used the same principle to further develop a quantifiable assay for association of fragments with native membranes. As shown in Fig. 5 A, membrane association of GFP-PH and of [170–320]-GFP are each specifically reduced only when the m1 receptor is expressed and activated by ACh.

Predicted Secondary Structure of Kir Channel COOH Terminus

Several different Kir COOH termini (Huang et al., 1998; Shyng and Nichols, 1998; Zhang et al., 1999; Shyng et al., 2000; Soom et al., 2001) have been shown to interact with membrane lipids, and homologous residues controlling PIP₂ sensitivity have been identified in different channels (Baukrowitz et al., 1998b; Huang et al., 1998; Shyng and Nichols, 1998; Zhang et al., 1999; Shyng et al., 2000). Many of these assays are indirect, and cannot exclude the possibility that the interaction with the membrane lipid is mediated via an intermediate protein interaction. Nevertheless, together with the present results, the data suggest the presence of a common KIRLI domain formed from the conserved sequence in the channel COOH terminus.

We performed predictions of the secondary structure of Kir channels using alignments of the conserved \sim 150 amino acids following M2 (residues 170–320 in Kir6.2, see Fig. 6 A). Following from the end of the M2 helix (predicted to end at \sim 173), multiple β -strands and a COOH-terminal α -helix (residues 306–311) are predicted, a structural arrangement that is common to lipid-interacting PH domains (Rebecchi and Scarlata, 1998). PH domains invariably contain seven major antiparallel β -strands with a COOH-terminal α -helix that contributes to stability of the domain structure. Importantly, the COOH-terminal helix is the only region in PH domains over which primary sequence homology is typically found. The conserved Trp and the large hydrophobic residue at position +4 interact with hydrophobic amino acids in the β -sheets to provide stability to the whole PH domain (Pitcher et al., 1995; Touhara et al., 1995), and mutations of these residues severely impair PH domain function (Shaw, 1996; Fushman et al., 1998). Upstream negative charges and no helix-breaking prolines are also features of the PH domain C-helix, and these features are all present in the 307–315 region of Kir channels (Fig. 3 A). The data obtained from systematic mutation are consistent with this region being α -helical in Kir6.2, such that mutations on one face abolish channel activity (Fig. 2). The conserved W311 and F315 residues, located on this face, are not only critical for channel activity in the full-length construct (Fig. 3), but also for stability and membrane association of isolated COOH-terminal fragments (Fig. 4). We suggest that this helix may play a

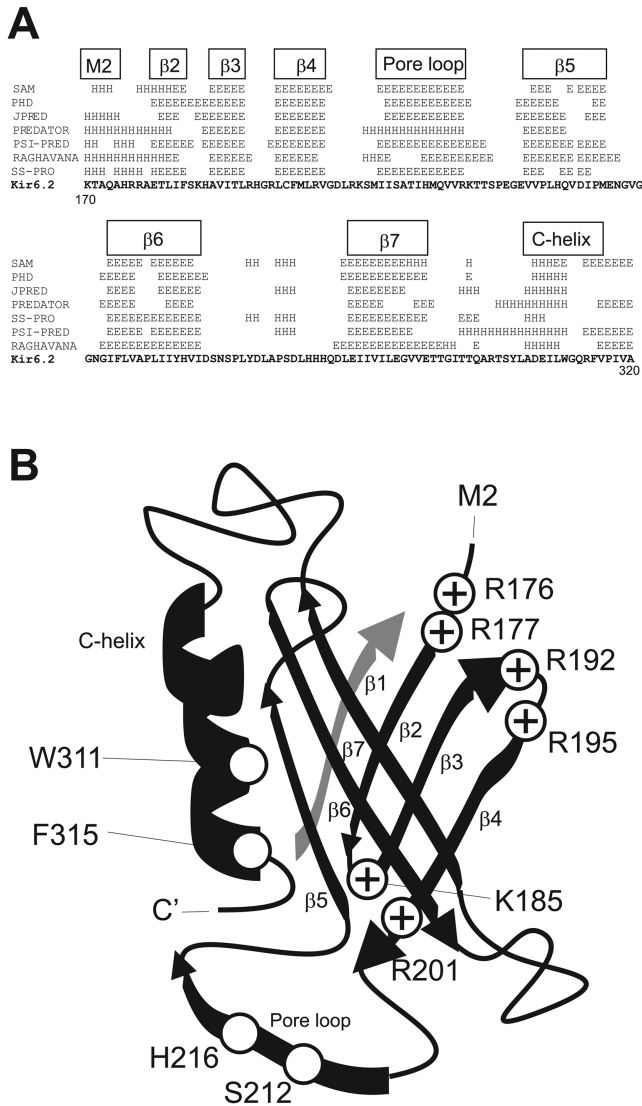


FIGURE 6. Predicted structure of conserved Kir cytoplasmic domain. (A) (above sequence) Computer-predicted secondary structures (E, β -strand, H, helix) of conserved COOH-terminal (residues 170–324) regions of Kir6.2 using multiple alignments with other inward rectifiers, generated by various algorithms indicated on left. The boxed indicators above correspond to the suggested secondary structures in B. (B) Cartoon ribbon diagram of suggested structural arrangement of the Kir6.2 KIRLI domain (see DISCUSSION).

structural role in the integrity of the KIRLI domain, analogous to the COOH-terminal α -helix of PH domains.

A Possible Structure of the KIRLI Domain

Given the functional and structural similarities between the KIRLI domain and established PH domains, we have explored the possibility that the tertiary structure of the KIRLI domain might be similar to that of the PH domain. In the absence of any structural information,

this speculative exercise may help to suggest further experiments. Fig. 6 B shows a cartoon illustrating a possible folding arrangement for the Kir6.2 COOH terminus cytoplasmic domain that would maintain an overall tertiary structure similar to that of the PH domain¹ (two antiparallel β -sheets and COOH-terminal α -helix). The arrangement is of a “pocket” formed by the two β -sheets (residues 176–201 and 220–290) with the C-helix (residues 307–315) forming a “hinge” between the two sheets, W311 and F315 side-chains being predicted to protrude into the “pocket” (Rebecchi and Scarlata, 1998). There is now considerable evidence for interaction of the conserved regions of the NH₂ terminus with the COOH terminus (Tinker et al., 1996; Koster et al., 1998; Tucker and Ashcroft, 1999) of Kir channels. This data may be reconciled by the suggestion that the conserved region of the NH₂ terminus normally generates the β 1 strand of the putative PH-like domain. Using protein fragments, Jones et al. (2001) have recently identified segments of the Kir6.2 COOH terminus that interact with the isolated NH₂ terminus and defined three segments of interaction (residues 170–204, 214–222, and 279–323) that correspond to the same COOH-terminal regions identified by Soom et al. (2001) as important for binding to PIP₂. Our suggested model includes critical roles for each of these regions in forming the KIRLI domain.

Charged residues clustered on one side of the “ β -pocket” in the β 1/ β 2 and β 3/ β 4 loops of PH domains are involved in interaction with PIP₂ (Harlan et al., 1995; Rebecchi and Scarlata, 1998; Soisson et al., 1998; Yagisawa et al., 1998; Carman et al., 2000). Systematic mutagenesis of Kir6.2 COOH-terminal-positive charges identified several residues (R176, R177, R192, R195) with significant effects on PIP₂ sensitivity when neutralized (Shyng et al., 2000), consistent with them mediating electrostatic interactions with the negatively charged phosphates of PIP₂. As shown in Fig. 6 B, placing the predicted COOH-terminal β -strands in the β 2- β 7 strand positions of the PH domain would cluster these critical residues in the β 1/ β 2 and β 3/ β 4 loops, i.e., on one side of the “ β -pocket”. The R176 and R177 residues are conserved in other Kir channels, and neutralization of the equivalent K188 and R189 in Kir2.1 (Soom et al., 2001), or R188 in Kir1.1 (Huang et al., 1998) also reduces PIP₂ sensitivity of channel activity.

Much evidence supports the idea of a negative allosteric interaction between PIP₂ and ATP in regulating the activity of Kir6.2 channels (Baukrowitz et al., 1998a; Shyng and Nichols, 1998; Enkvetchakul et al.,

¹Stable models of the KIRLI domain (unpublished data) can be generated by threading the Kir COOH terminus threaded onto the PH domain of pleckstrin as a template (Guex and Peitsch, 1997), but uncertainty in the correct alignment reduces the likely value of such models.

2000), with functional competition between the two ligands, but separate binding sites. Several residues have emerged as candidate ATP binding residues (Drain et al., 1998; Gribble et al., 1998; Tucker et al., 1998; Shyng et al., 2000), and mutation of certain charged residues has large effects on ATP sensitivity, but minimal effects on PIP₂ sensitivity. Intriguingly, two of these residues, K185 (Tucker et al., 1998) and R201 (Shyng et al., 2000), are located within the proposed KIRLI domain, and would be predicted to lie at the opposite side of the “β-pocket” to the PIP₂-interacting residues (Fig. 6 B), consistent with the above hypothesis.

In addition to the computer-predicted β2-β7 strands and C-helix of Kir channels (Fig. 6 A), there is a region between putative β4 and β5 that is predicted to be either α-helix or β-strand (Fig. 6). Several studies on various Kir channels indicate that residues in this region actually form part of the cytoplasmic entrance to the pore. In Kir2.1, E224 (S212 in Kir6.2) controls the affinity for pore-blocking polyamines (Yang et al., 1995) and H216 in Kir6.2 controls the pH-dependence of polyamine block (Baukrowitz et al., 1999). Cysteine-substituents of several residues within this region of Kir2.1 are modified by methyl-thio-sulphydryl reagents, indicating water accessibility (Lu et al., 1999). PH domains can tolerate large insertions in the loops between β-strands (Rebecchi and Scarlata, 1998), and we propose that this region may form an insertion that lines the cytoplasmic entrance to the pore. As such it is likely to contribute to the control of open state stability of the channel, and, as with the M2 transmembrane segment (Enkvetchakul et al., 2000), mutations within it will affect open state stability and apparent PIP₂ sensitivity of channel activity. Accordingly, neutralization of R206 or K222 in this region of Kir6.2 (Shyng et al., 2000), or mutations of residues in the equivalent 207–245 segment of Kir2.1 (Zhang et al., 1999), reduce apparent PIP₂ sensitivity.

PIP₂ activation is common to all Kir channels, suggesting a conserved mechanism of interaction. Previous studies have demonstrated direct interaction of isolated COOH-terminal fragments with PIP₂ (Huang et al., 1998; Soom et al., 2001). We now demonstrate that the conserved COOH terminus (residues 170–320) associates with cellular membranes, and this association can be regulated by PLC activation and PIP₂ hydrolysis. Systematic mutagenesis of Kir6.2 has identified positive charges in this domain that when mutated alter PIP₂ sensitivity (Shyng et al., 2000); neutralization of any of the basic residues beyond the domain are without effect on channel activity (Shyng et al., 2000). Systematic alanine scanning now indicates the likely presence of a COOH-terminal α-helix; mutation of conserved Trp and Phe residues in this segment both abolish channel activity and interfere with domain expression and

membrane association. The data provide evidence for the existence of a conserved KIRLI domain that is critically involved in physiological regulation of channel activity.

We are very grateful to Show-Ling Shyng for help with preliminary studies and constructs, and to Diomedes Logothetis and colleagues for m1 and m2 receptor clones.

This work was primarily supported by National Institutes of Health grant HL54171 (to C.G. Nichols), as well as by a National Institutes of Health Cardiovascular Training grant (Fellowship support of C.A. Cukras), and the Washington University National Institutes of Health DRTC (DK20579).

REFERENCES

- Baukrowitz, T., U. Schulte, D. Oliver, S. Herlitze, T. Krauter, S.J. Tucker, J.P. Ruppersberg, and B. Fakler. 1998a. PIP₂ and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science*. 282: 1141–1144.
- Baukrowitz, T., U. Schulte, D. Oliver, S. Herlitze, T. Krauter, S.J. Tucker, J.P. Ruppersberg, and B. Fakler. 1998b. PIP₂ and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science*. 282: 1141–1144.
- Baukrowitz, T., S.J. Tucker, U. Schulte, K. Benndorf, J.P. Ruppersberg, and B. Fakler. 1999. Inward rectification in K_{ATP} channels: a pH switch in the pore. *EMBO J.* 18:847–853.
- Carman, C.V., L.S. Barak, C. Chen, L.Y. Liu-Chen, J.J. Onorato, S.P. Kennedy, M.G. Caron, and J.L. Benovic. 2000. Mutational analysis of Gbetagamma and phospholipid interaction with G protein-coupled receptor kinase 2. *J. Biol. Chem.* 275:10443–10452.
- Caulfield, M.P., and N. Birdsall. 1998. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* 50:279–290.
- Cifuentes, M.E., T. Delaney, and M.J. Rebecchi. 1994. D-myo-inositol 1,4,5-trisphosphate inhibits binding of phospholipase C-delta 1 to bilayer membranes. *J. Biol. Chem.* 269:1945–1948.
- Doyle, D.A., J.M. Cabral, R.A. Pfoetzner, A. Kuo, J.M. Gulbis, S.L. Cohen, B.T. Chait, and R. MacKinnon. 1998. The structure of the potassium channel: molecular basis of K⁺ conduction and selectivity. *Science*. 280:69–77.
- Drain, P., L. Li, and J. Wang. 1998. K_{ATP} channel inhibition by ATP requires distinct functional domains of the cytoplasmic C terminus of the pore-forming subunit. *Proc. Natl. Acad. Sci. USA.* 95: 13953–13958.
- Enkvetchakul, D., G. Loussouarn, E. Makhina, S.L. Shyng, and C.G. Nichols. 2000. The kinetic and physical basis of K(ATP) channel gating: toward a unified molecular understanding. *Biophys. J.* 78: 2334–2348.
- Fakler, B., J.H. Schultz, J. Yang, U. Schulte, U. Brandle, H.P. Zenner, L.Y. Jan, and J.P. Ruppersberg. 1996. Identification of a titratable lysine residue that determines sensitivity of kidney potassium channels (ROMK) to intracellular pH. *EMBO J.* 15:4093–4099.
- Fan, Z., and J.C. Makielski. 1997. Anionic phospholipids activate ATP-sensitive potassium channels. *J. Biol. Chem.* 272:5388–5395.
- Furukawa, T., T. Yamane, T. Terai, Y. Katayama, and M. Hiraoka. 1996. Functional linkage of the cardiac ATP-sensitive K⁺ channel to the actin cytoskeleton. *Pflügers Arch.* 431:504–512.
- Fushman, D., T. Najmabadi-Haske, S. Cahill, J. Zheng, H. LeVine III, and D. Cowburn. 1998. The solution structure and dynamics of the pleckstrin homology domain of G protein-coupled receptor kinase 2 (beta-adrenergic receptor kinase 1). A binding partner of Gbetagamma subunits. *J. Biol. Chem.* 273:2835–2843.
- Gribble, F.M., S.J. Tucker, T. Haug, and F.M. Ashcroft. 1998.

- MgATP activates the beta cell K_{ATP} channel by interaction with its SUR1 subunit. *Proc. Natl. Acad. Sci. USA*. 95:7185–7190.
- Gueux, N., and M.C. Peitsch. 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis*. 18:2714–2723.
- Harlan, J.E., H.S. Yoon, P.J. Hajduk, and S.W. Fesik. 1995. Structural characterization of the interaction between a pleckstrin homology domain and phosphatidylinositol 4,5-bisphosphate. *Biochemistry*. 34:9859–9864.
- Harvey, J., S.C. Hardy, A.J. Irving, and M.L. Ashford. 2000. Leptin activation of ATP-sensitive K^+ (K_{ATP}) channels in rat CRI-G1 insulinoma cells involves disruption of the actin cytoskeleton. *J. Physiol.* 527:95–107.
- Hilgemann, D.W., and R. Ball. 1996. Regulation of cardiac Na^+ , Ca^{2+} exchange and K_{ATP} potassium channels by PIP₂. *Science*. 273:956–959.
- Hilgemann, D.W., S. Feng, and C. Nasuhoglu. 2001. The complex and intriguing lives of PIP₂ with ion channels and transporters. *Sci STKE*. 2001:RE19.
- Hodgkin, M.N., M.R. Masson, D. Powner, K.M. Saqib, C.P. Ponting, and M.J. Wakelam. 2000. Phospholipase D regulation and localisation is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain. *Curr. Biol.* 10:43–46.
- Huang, C.L., S. Feng, and D.W. Hilgemann. 1998. Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gbetagamma. *Nature*. 391:803–806.
- Huang, C.L., P.A. Slesinger, P.J. Casey, Y.N. Jan, and L.Y. Jan. 1995. Evidence that direct binding of G beta gamma to the GIRK1 G protein-gated inwardly rectifying K^+ channel is important for channel activation. *Neuron*. 15:1133–1143.
- Inagaki, N., T. Gonoi, J.P. Clement, N. Namba, J. Inazawa, G. Gonzalez, L. Aguilar-Bryan, S. Seino, and J. Bryan. 1995. Reconstitution of IK_{ATP} : an inward rectifier subunit plus the sulfonylurea receptor. *Science*. 270:1166–1170.
- Janmey, P.A. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.* 56:169–191.
- Jones, P.A., S.J. Tucker, and F.M. Ashcroft. 2001. Multiple sites of interaction between the intracellular domains of an inwardly rectifying potassium channel, Kir6.2. *FEBS Lett.* 508:85–89.
- Kobrinsky, E., T. Mirshahi, H. Zhang, T. Jin, and D.E. Logothetis. 2000. Receptor-mediated hydrolysis of plasma membrane messenger PIP₂ leads to K^+ -current desensitization. *Nat. Cell Biol.* 2:507–514.
- Koster, J.C., K.A. Bentle, C.G. Nichols, and K. Ho. 1998. Assembly of ROMK1 (Kir 1.1a) inward rectifier K^+ channel subunits involves multiple interaction sites. *Biophys. J.* 74:1821–1829.
- Lemmon, M.A., K.M. Ferguson, R. O'Brien, P.B. Sigler, and J. Schlessinger. 1995. Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proc. Natl. Acad. Sci. USA*. 92:10472–10476.
- Lemmon, M.A., K.M. Ferguson, and J. Schlessinger. 1996. PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*. 85:621–624.
- Liou, H.H., S.S. Zhou, and C.L. Huang. 1999. Regulation of ROMK1 channel by protein kinase A via a phosphatidylinositol 4,5-bisphosphate-dependent mechanism. *Proc. Natl. Acad. Sci. USA*. 96:5820–5825.
- Liu, Y., M. Holmgren, M.E. Jurman, and G. Yellen. 1997. Gated access to the pore of a voltage-dependent K^+ channel. *Neuron*. 19:175–184.
- Loussouarn, G.P., L.R. Masia, R. Rose, and T. Nichols. 2001. Flexibility of the Kir6.2 inward rectifier K^+ channel pore. *Proc. Natl. Acad. Sci. USA*. 98:4227–4232.
- Lu, T., Y.G. Zhu, and J. Yang. 1999. Cytoplasmic amino and carboxyl domains form a wide intracellular vestibule in an inwardly rectifying potassium channel. *Proc. Natl. Acad. Sci. USA*. 96:9926–9931.
- Ma, D., N. Zerangue, Y.F. Lin, A. Collins, M. Yu, Y.N. Jan, and L.Y. Jan. 2001. Role of ER export signals in controlling surface potassium channel numbers. *Science*. 291:316–319.
- Makhina, E.N., and C.G. Nichols. 1998. Independent trafficking of K_{ATP} channel subunits to the plasma membrane. *J. Biol. Chem.* 273:3369–3374.
- Nagel, W., P. Schilcher, L. Zeitlmann, and W. Kolanus. 1998. The PH domain and the polybasic c domain of cytohesin-1 cooperate specifically in plasma membrane association and cellular function. *Mol. Biol. Cell*. 9:1981–1984.
- Nichols, C.G., and A.N. Lopatin. 1997. Inward rectifier potassium channels. *Annu. Rev. Physiol.* 59:171–191.
- Pearson, W.L., M. Dourado, M. Schreiber, L. Salkoff, and C.G. Nichols. 1999. Expression of a functional Kir4 family inward rectifier K^+ channel from a gene cloned from mouse liver. *J. Physiol.* 514:639–653.
- Perozo, E., D.M. Cortes, and L.G. Cuello. 1999. Structural rearrangements underlying K^+ -channel activation gating. *Science*. 285:73–78.
- Pitcher, J.A., K. Touhara, E.S. Payne, and R.J. Lefkowitz. 1995. Pleckstrin homology domain-mediated membrane association and activation of the beta-adrenergic receptor kinase requires coordinate interaction with G beta gamma subunits and lipid. *J. Biol. Chem.* 270:11707–11710.
- Rebecchi, M.J., and S. Scarlata. 1998. Pleckstrin homology domains: a common fold with diverse functions. *Annu. Rev. Biophys. Biomol. Struct.* 27:503–528.
- Reuveny, E., P.A. Slesinger, J. Inglese, J.M. Morales, J.A. Iniguez-Lluhi, R.J. Lefkowitz, H.R. Bourne, Y.N. Jan, and L.Y. Jan. 1994. Activation of the cloned muscarinic potassium channel by G protein beta gamma subunits. *Nature*. 370:143–146.
- Rohacs, T., J. Chen, G.D. Prestwich, and D.E. Logothetis. 1999. Distinct specificities of inwardly rectifying K^+ channels for phosphoinositides. *J. Biol. Chem.* 274:36065–36072.
- Shaw, G. 1996. The pleckstrin homology domain: an intriguing multifunctional protein module. *Bioessays*. 18:35–46.
- Shyng, S.L., C.A. Cukras, J. Harwood, and C.G. Nichols. 2000. Structural determinants of PIP(2) regulation of inward rectifier K^+ (ATP) channels. *J. Gen. Physiol.* 116:599–608.
- Shyng, S.L., and C.G. Nichols. 1998. Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. *Science*. 282:1138–1141.
- Soisson, S.M., A.S. Nimnual, M. Uy, D. Bar-Sagi, and J. Kuriyan. 1998. Crystal structure of the Dbl and pleckstrin homology domains from the human Son of sevenless protein. *Cell*. 95:259–268.
- Soom, M., R. Schonherr, Y. Kubo, C. Kirsch, R. Klinger, and S.H. Heinemann. 2001. Multiple PIP₂ binding sites in Kir2.1 inwardly rectifying potassium channels. *FEBS Lett.* 490:49–53.
- Stauffer, T.P., S. Ahn, and T. Meyer. 1998. Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P₂ concentration monitored in living cells. *Curr. Biol.* 8:343–346.
- Tinker, A., Y.N. Jan, and L.Y. Jan. 1996. Regions responsible for the assembly of inwardly rectifying potassium channels. *Cell*. 87:857–868.
- Touhara, K., W.J. Koch, B.E. Hawes, and R.J. Lefkowitz. 1995. Mutational analysis of the pleckstrin homology domain of the beta-adrenergic receptor kinase. Differential effects on G beta gamma and phosphatidylinositol 4,5-bisphosphate binding. *J. Biol. Chem.* 270:17000–17005.
- Tucker, S.J., and F.M. Ashcroft. 1999. Mapping of the physical interaction between the intracellular domains of an inwardly rectify-

- ing potassium channel, Kir6.2. *J. Biol. Chem.* 274:33393–33397.
- Tucker, S.J., F.M. Gribble, P. Proks, S. Trapp, T.J. Ryder, T. Haug, F. Reimann, and F.M. Ashcroft. 1998. Molecular determinants of K_{ATP} channel inhibition by ATP. *EMBO J.* 17:3290–3296.
- Tucker, S.J., F.M. Gribble, C. Zhao, S. Trapp, and F.M. Ashcroft. 1997. Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature.* 387:179–183.
- Varnai, P., and T. Balla. 1998. Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* 143:501–510.
- Varnai, P., K.I. Rother, and T. Balla. 1999. Phosphatidylinositol 3-kinase-dependent membrane association of the Bruton's tyrosine kinase pleckstrin homology domain visualized in single living cells. *J. Biol. Chem.* 274:10983–10989.
- Wang, X., A.J. Levi, and A.P. Halestrap. 1996. Substrate and inhibitor specificities of the monocarboxylate transporters of single rat heart cells. *Am. J. Physiol.* 270:H476–H484.
- Xie, L.H., M. Horie, and M. Takano. 1999. Phospholipase C-linked receptors regulate the ATP-sensitive potassium channel by means of phosphatidylinositol 4,5-bisphosphate metabolism. *Proc. Natl. Acad. Sci. USA.* 96:15292–15297.
- Yagisawa, H., K. Sakuma, H.F. Paterson, R. Cheung, V. Allen, H. Hirata, Y. Watanabe, M. Hirata, R.L. Williams, and M. Katan. 1998. Replacements of single basic amino acids in the pleckstrin homology domain of phospholipase C-delta1 alter the ligand binding, phospholipase activity, and interaction with the plasma membrane. *J. Biol. Chem.* 273:417–424.
- Yang, J., Y.N. Jan, and L.Y. Jan. 1995. Control of rectification and permeation by residues in two distinct domains in an inward rectifier K^+ channel. *Neuron.* 14:1047–1054.
- Yang, Z., and C. Jiang. 1999. Opposite effects of pH on open-state probability and single channel conductance of kir4.1 channels. *J. Physiol.* 520:921–927.
- Zerangue, N., B. Schwappach, Y.N. Jan, and L.Y. Jan. 1999. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron.* 22:537–548.
- Zhang, H., C. He, X. Yan, T. Mirshahi, and D.E. Logothetis. 1999. Activation of inwardly rectifying K^+ channels by distinct PtdIns(4,5)P₂ interactions. *Nat. Cell Biol.* 1:183–188.