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C-Terminal Determinants of Kir4.2 Channel Expression

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Abstract. Inward rectifier potassium (Kir) channels serve important functional and modulatory roles in a wide variety of cells. While the activity of several members of this channel family are tightly regulated by intracellular messengers such as adenosine triphosphate, G proteins, protein kinases and pH, other members are tonically active and activity is controlled only by the expression level of the protein. In a number of Kir channels, sequence motifs have been identified which determine how effectively the channel is trafficked to and from the plasma membrane. In this report, we identify a number of trafficking determinants in the Kir4.2 channel. Using mutational analysis, we found that truncation of the C terminus of the protein increased current density in *Xenopus* oocytes, although multiple mutations of the C terminus had no effect on current density. Instead, mutation of a unique region of the channel significantly increased current density. Selective mutation of a putative tyrosine phosphorylation site within this region mimicked the increase in current, suggesting that tyrosine phosphorylation of the protein increases channel retrieval from the membrane (or prevents trafficking to the membrane). Mutation of a previously identified trafficking determinant, K110N, also caused an increase in current density, and combining these mutations caused a multiplicative increase in current, suggesting that these two mutations increase current by independent mechanisms. These data demonstrate novel determinants of Kir4.2 channel expression.

Key words: Endoplasmic reticulum — Oocyte — Tyrosine phosphorylation — Trafficking

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Introduction

For living cells to function optimally, the flow of different ions across the plasma membrane must be tightly controlled. Ion transport proteins (pumps) actively move molecules across the plasma membrane to create electrochemical gradients, while other transport proteins and channels permit passive flow of molecules/ions to dissipate these gradients. Any mismatch between pump and channel activity could lead to deleterious changes in cell volume, membrane potential or Ca^{2+} handling. Net cellular ion flow, through a particular type of ion channel, can be described by the equation $G = g * P_O * N$, where G is net cellular conductance, g is the unit conductance of a single channel, P_O is the single-channel open probability and N is the total number of channels in the cell's plasma membrane. Ion flow can be modulated by changing any of these three parameters but most commonly occurs from changes in P_O and N. Rapid changes in G are most easily accomplished by changes in P_O (e.g., voltage gating, agonist gating such as binding of acetylcholine to the nACh receptor), whereas slower and more persistent changes in G can be achieved by changing N, i.e., by changing the number of active channels in the plasma membrane.

The processes which control delivery and withdrawal of ion channels to the plasma membrane are many, and these pathways are still being characterized. The earliest steps in the pathway consist of regulation of gene transcription and RNA translation. Once the proteins are synthesized and transported into the endoplasmic reticulum (ER), the rate at which proteins reach the plasma membrane is determined by their interactions with a large set of processing proteins as well as interactions with accessory subunits. While the mechanisms that control the optimal number of channels in the plasma membrane remain obscure, it is clear that defects in

channel trafficking to the membrane can be pathological (Yan et al., 2004).

Recent years have seen a number of studies on the trafficking of inward rectifier K^+ channel (Kir) proteins in heterologous expression systems and in diseases. The earliest findings were that certain Kir proteins were not efficiently expressed without the appropriate accessory subunits (e.g., Kir3.1 requires Kir3.2 or Kir3.4 [Krapivinsky et al., 1995], Kir6.2 requires SUR1 or SUR2 [Inagaki et al., 1995]). In the case of Kir6.2, it was found that C-terminal (and N-terminal) truncations produced functional ion channels in the absence of the sulfonylurea receptor (SUR) (Tucker et al., 1997) and that a short C-terminal sequence prevented plasmalemma expression and promoted ER retention of homomeric Kir6.2 proteins not complexed with SUR (Zerangue et al., 1999). Other trafficking signals have been identified in Kir1.1 and Kir2 channels, which promote export of the channels from the ER (Ma et al., 2001), as well as in Kir3 (Ma et al., 2002). Little has been reported about trafficking of the four remaining Kir channels: Kir4.1, Kir4.2, Kir5.1 and Kir7.1. Reports on the initial cloning of Kir4.2 (first called "Kir1.3" [Shuck et al., 1997]) indicated that the channel was not functional, while later reports showed low levels of channel activity, activity when coexpressed with Kir5.1 and increased levels of expression when a single residue in the first extracellular domain was mutated to correspond to the homologous residue in Kir1.1 (K110N) (Derst et al., 1998; Pearson et al., 1999). Kir5.1 was initially reported to be nonfunctional when expressed alone and functional only when coexpressed with Kir4 channels (Bond et al., 1994; Pessia et al., 1996). Kir5.1 is successfully transported to the plasma membrane when expressed without Kir4 subunits (Tanemoto et al., 2002). The possibility that Kir5.1 requires an accessory subunit has received little attention, but there is recent evidence that coexpression of Kir5.1 with a PDZ protein, PSD-95, generates functional channels (Tanemoto et al., 2002). In addition to signals which control export to the plasma membrane, other sequence motifs direct proteins to a specific pole or subregion of a cell. Many Kir channels contain PDZ binding motifs at their distal C termini, including Kir1.1, Kir2, Kir3.2 and Kir4. Kir4. In Kir4.1, this domain promotes channel clustering when coexpressed with the PDZ protein PSD-95 (Horio et al., 1997).

In this study, we add to the list of Kir transport signals by showing that C-terminal truncations of Kir4.2 increase membrane currents and that predicted signal motifs in the C terminus are not responsible for this increase in current amplitude. Instead, a novel C-terminal sequence is responsible for low expression levels of Kir4.2. Mutating or deleting a putative tyrosine phosphorylation motif in this region causes substantial increases in whole-cell current amplitude. As Kir4.2 channels have a high basal P_O (~0.5–0.9) (Derst et al., 1998; Pessia et al., 2001), the observed increase in current (greater than fivefold) cannot be attributed to increases in P_O and is likely due to increases in N caused by a change in trafficking kinetics. Further, deletion of this C-terminal sequence increases current levels in a manner that is independent of current increases caused by the K110N mutation, indicating that multiple pathways exist in regulating Kir4.2 trafficking to the plasma membrane.

Materials and Methods

Molecular Biology

All experiments were done using mKir4.2 cDNA cloned into the vector pBscmxt, a pBluescript derivative, as described previously (Pearson et al., 1999). Mutations and truncations were introduced into the coding sequence using the overlap extension polymerase chain reaction (PCR) technique. The resulting mutated PCR products were ligated back into the vector and propagated in DH5α cells. Recombinant plasmid DNA was recovered from 100–250 ml bacterial preps using Promega (Madison, WI) Wizard Prep kits. The veracity of the DNA sequence of each mutant construct was confirmed by sequencing the entire mutant cassette. cRNA for oocyte injection was produced by *in vitro* transcription. Briefly, DNA was linearized with either *Hin*cII or *Sal*I. This reaction was then treated with protease K for 1 h at 50°C, extracted with phenol/chloroform/isoamyl alcohol to remove contamination by RNase, precipitated with ammonium acetate and ethanol and finally resuspended in DNase/RNase-free water. The transcription reaction was performed using T3 RNA polymerase for 2 h at 37°C in the presence of RNase inhibitor and terminated by incubating with DNase for 10 min. The RNA was then further purified by two rounds of sodium acetate/ethanol precipitation. RNA was quantified by spectrophotometer and by ethidium bromide fluorescence of aliquots run on a denaturing agarose gel.

Animals

Xenopus laevis were maintained in an aquarium and cared for according to a protocol approved by Washington University's Animal Care Committee. Oocytes were obtained by partial ovariectomy under tricaine anesthesia. Oocytes were defolliculated by incubating in "0 Ca ND96" solution (see below) containing 1.5 mg/ml collagenase (type 1a) with gentle incubation for ~1 h. After collagenase treatment, oocytes were rinsed with 0 Ca ND96, typically incubated in 100 mM potassium phosphate (pH 5.2) for a brief period (1–2 min) to aid in removing the follicular layer and finally stored in 1.8 mM Ca ND96 containing penicillin and streptomycin.

RNA was injected into oocytes through beveled glass capillaries, using either a pressure ejection system or a positive displacement injection pipette. Injection volumes were typically 50–100 nl, with RNA concentrations typically 0.2 μg/μl (or less). For each set of injections, care was taken to assure that each oocyte was injected with very similar amounts of DNA.

Electrophysiology

Two-electrode voltage clamp was performed with a Warner (Hamden, CT) OC-725C voltage-clamp amplifier, controlled by

pClamp software (Axon Instruments, Foster City, CA). Amplifier sense and ground electrodes were attached to the bath through 3 M KCl agar bridges. Recording electrodes were pulled from borosilicate glass on a Flaming-Brown P97 electrode puller (Sutter Instruments, Novato, CA) and filled with 3 M KCl. Electrodes initially had resistance of < 1 MΩ. Recordings were typically made 2–3 days after oocytes were injected with RNA. Oocytes were placed on an elevated platform in a Plexiglas recording chamber, which allows free flow of solution around all sides of the oocyte. The recording chamber volume was ~0.5 ml, with good laminar flow properties. Solutions were delivered to the recording chamber from sealed reservoirs through multiple switched lines that enter at the head of the recording chamber. Flow rate was set by a peristaltic pump delivering air to the sealed reservoir. This recording chamber allowed rapid turnover of the bath solution so that at the flow rates used (~1 ml/min) solution changes at the oocyte were complete in < 30 s after switching.

Solutions

All solutions were based on "ND96" solution: 1.8 mM ND96 containing 96 mM NaCl, 2 mM KCl, 5 mM Na-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mM $MgCl_2$, 1.8 mM $CaCl_2$ (pH 7.5). Solutions used for electrophysiological recordings (0 Ca ND96) were based on the above formula but contained no added Ca^{2+}. K^+ solution (2 mM) contained 96 mM NaCl, 2 mM KCl, 5 mM Na-HEPES, 1 mM $MgCl_2$ (pH 7.5). K^+ solution (100 mM) contained 98 mM KCl, 1 mM K-HEPES, 5 mM $MgCl_2$ (pH 7.5). Intermediate K^+ concentrations were obtained by mixing the appropriate amounts of 2 mM K^+ and 0 Ca ND96 together. For experiments in which 100 μM Ba^{2+} was used to block Kir current, 1 M $BaCl_2$ was added to the appropriate physiological solution.

Data Analysis

Expression levels of Kir channels were compared by two different methods. When comparing data from several different sets of oocyte injections, current measured from mutant channel constructs was normalized to current from wild-type channels recorded on the same day, and the data from several days were pooled for analysis. Leakage and background currents were subtracted by measuring the increase in current at −100 mV ($\sim E_K$ for 2 mM K^+) after the $[K^+]$ was raised from 2 mM, typically 100 mM. Alternatively, Kir current was quantified by measuring the amplitude of current blocked by 100 μM Ba at a negative potential (typically −140 mV).

Results

In order to explore the possibilities that specific sequences in the nonconserved cytoplasmic domain of Kir4.2 channels determine channel expression, a panel of mutant Kir4.2 channels was expressed in *Xenopus* oocytes. The mutant channels contained either arbitrary deletions of C-terminal regions of the protein or mutations of potential trafficking motifs. The primary structures of the wild-type and mutant proteins are shown in Figure 1, with potential determinants of protein trafficking indicated. In particular, mutation Q371st deleted the putative PDZ binding motif at the distal C terminus (SNV) (Horio et al., 1997); mutation L369E disrupted a potential dileucine targeting motif, D/E-$(X)_{3-5}$-L-L (Pond et al., 1995); mutation R363G disrupted a sequence with superficial resemblance to R-K-R or R-X-R or dibasic ER retention motifs (Zerangue et al., 1999, 2001). Currents from the resulting channels were measured by two-electrode voltage clamp. Mutant channels produced whole-cell currents that were biophysically indistinguishable from wild-type channels in terms of K selectivity and voltage sensitivity/rectification (*data not shown*). Also, all mutant channels retained K regulation (i.e., current amplitude slowly increased in the presence of elevated $[K^+]_{Out}$) (Pearson et al., 1999) (*data not shown*). In channels with deletions or mutations in the distal 16 amino acids, changes in current density were negligible (Fig. 2). However, in channels with greater deletions of the C terminus (22 amino acids), current amplitudes were increased substantially (Fig. 2). In particular, Q354st and C339st produced currents roughly 10-fold larger than wild-type. Since the Q360st truncation did not affect current amplitudes while the Q354st truncation produced substantial increases in current, a nine-amino acid internal deletion mutant, Δ354–362 (QYRQEDQRE), was constructed to further explore the role of this region of the protein responsible for increasing current amplitudes. Currents from Δ354–362 channels were also substantially larger than currents from wild-type channels, implying that residues within this region are specifically negative regulators of channel density.

Close inspection of the sequence in the region of amino acids 354–362 showed that a potential tyrosine phosphorylation motif, K-$(X)_{2-3}$-E-$(X)_2$-Y, was present. Since deletion of this region increased current amplitude, we hypothesized that a phosphotyrosine at this location was somehow decreasing the current amplitude in wild-type channels and in channels with mutations distal to the phosphorylation motif. Indeed, a phosphotyrosine in the C terminus of Kir1.1 has been shown to decrease whole-cell current by increasing endocytosis of the channel (Moral et al., 2001; Sterling et al., 2002). To test this hypothesis, mutants of this residue were constructed. One mutation (Y355F) replaced the tyrosine with a phenylalanine, a structurally similar amino acid that cannot be phosphorylated. This substitution caused a very large increase in current amplitude above wild type (Fig. 3), similar to what was seen in the Q354st, C339st and Δ354–362 mutations. We additionally replaced tyrosine with aspartate (Y355D), which is negatively charged and may partially mimic the effect of phosphorylation (Lin et al., 2002a; Palmada et al., 2003). In contrast, to the neutralization mutation, Y355D produced currents that were a modest ~3.5-fold greater than wild type (Fig. 3). Together, these data argue that a phosphotyrosine at position 354 is likely responsible for retention of channels within the cell. Phosphorylation can introduce −3 charges; however, the Y- > D results in a fixed −1 net charge at

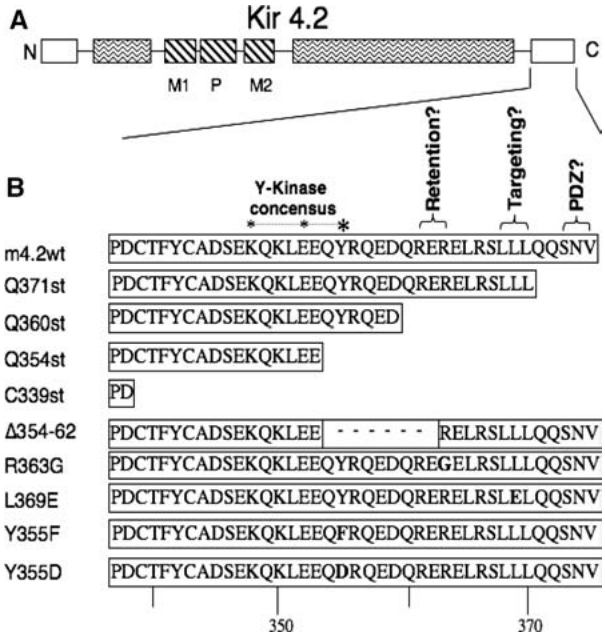

Fig. 1. Structure of mutant Kir4.2 channels. (*A*) Schematic representation of Kir4.2 channel protein, with different structural domains indicated by boxes. *Filled boxes* represent the core region of Kir channels, which show a high degree of structural homology across all eukaryotic Kir channels. *Hatched boxes* represent regions that form the transmembrane domains (M1, M2) and pore-helix/selectivity filter (P). *Boxes with wavy lines* indicate regions known to form the cytoplasmic pore domain in other Kir channels. *Open boxes* at the distal N and C termini indicate regions which are poorly conserved across channel families. (*B*) Expanded distal C terminus of Kir 4.2 and mutant constructs, with sequences shown. Also shown are sequences which resemble putative trafficking or targeting motifs found in other proteins. *Asterisks* denote residues defining a consensus sequence for tyrosine kinase phosphorylation.

this residue, and we speculate that the retention effect of the Y- > D mutation may not therefore be as great as that for a phosphotyrosine residue in the wild-type channel.

Previous reports have also indicated that wild-type Kir4.2 current density was low compared to currents from a mutant channel, K110N (Derst et al., 1998). Residue K110N is located not in the distal C terminus of the channel but in the extracellular region between the first transmembrane helix and the pore helix/selectivity filter region. Derst et al. (1998) found no difference between the P_O of K110N channels and wild-type channels and concluded that K110N current density was increased by increasing the number of active channels present in the plasma membrane. Given the high P_O of Kir4.2, C-terminal truncations identified in this study must also increase current by increasing the number of functional channels in the membrane. To explore the interaction of the K110N mutation with C-terminal truncations, we made double mutant channels combining the K110N mutation with C-terminal mutations. While the K110N mutation and C-terminal mutations individually increased current levels 5- to 10-fold, double mutant channels (e.g., K110N/Q354st) increased current levels ~25-fold (Fig. 4), consistent with the mechanisms of current increase being essentially independent.

Discussion

A theme which has emerged from structural studies of potassium channels is that the conserved core region of the channel, a region of ~275 amino acids, is primarily responsible for a channel's biophysical properties (P_O, g, gating) (Flagg et al., 2002; Leipziger et al., 2000; Schulte et al., 1999; Stanfield et al., 1994), while the distal regions of the protein are additionally involved in molecular interactions that affect cellular localization and trafficking of the channel protein (Cohen et al., 1996; Ishii et al., 1997; Kurschner et al., 1998; Ma et al., 2002; Tanemoto et al., 2002; Zerangue et al., 1999). While this understanding emerged from studies using techniques such as hydropathic analysis of channel sequences and site-directed mutagenesis coupled with electrophysiology, more recent studies employing X-ray crystallography have strengthened this understanding (Nishida & MacKinnon, 2002). In particular, Nishida and MacKinnon found that in Kir3.1 the conserved regions of the N and C termini form a tetrameric domain surrounding a cytoplasmic pore, while the distal N and C termini are outside this core domain.

The present study provides another example of a Kir channel whose level of expression is regulated by distal regions of the protein. The Kir4.2 channel was initially reported to be nonfunctional when

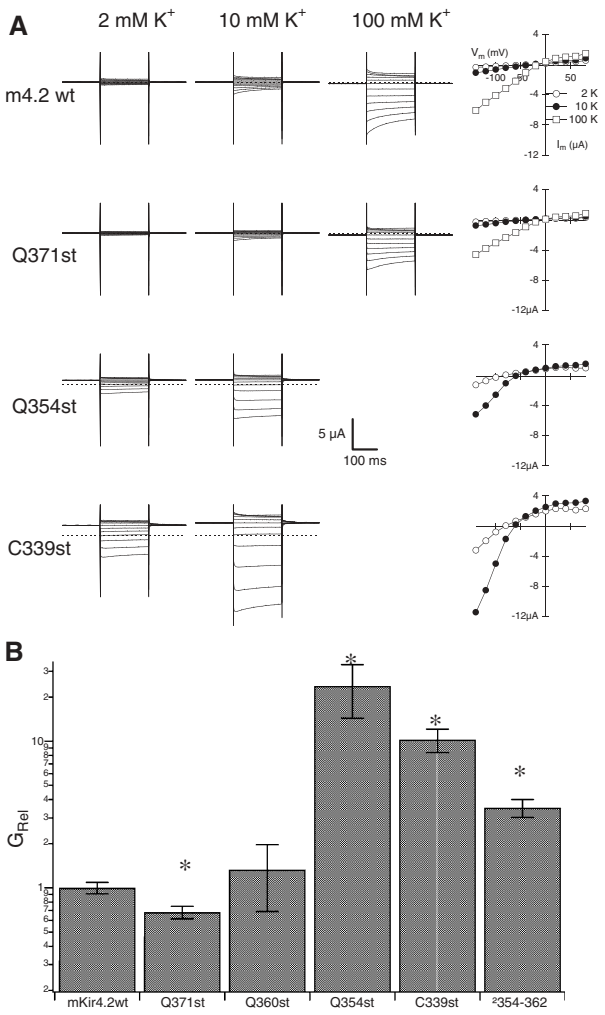

Fig. 2. C-terminal truncations increase current density. (*A*) Representative current traces and current-voltage plots from oocytes expressing wild-type or truncated Kir4.2 channels. Traces show current recorded in 2, 10 or 100 mM K^+ for channels expressing current at low density. For channels expressing current at high density, current levels were too great to record in 100 mM K^+ using the same voltage protocol. Cells were held at −20 m*V* and stepped to potentials between −140 and 80 m*V*. (*B*) Normalized conductance of oocytes expressing wild-type *vs.* mutant Kir channels. Note the logarithmic axis. Conductance was normalized to average wild-type current levels measured under the same conditions. *Asterisks* denote difference from wild-type is statistically significant ($P < 0.01$, except Q354st, $P = 0.023$) using Student's *t*-test.

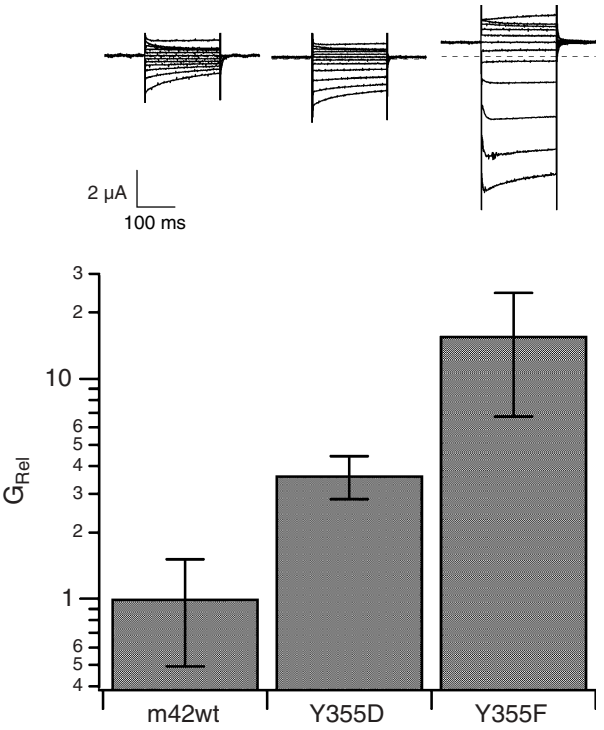

Fig. 3. Point mutation of Y335 affects current density. (*Top*) Representative traces of current from wild-type, Y355D and Y355F Kir4.2 mutations. Currents were recorded in 10 mM K^+, with cells held at −20 m*V* and stepped to potentials between −140 and 80 m*V*. (*Bottom*) Pooled data of relative conductance. Mutant channel current was statistically greater than wild-type in each case ($P < 0.05$, $n = 5$–6). Note the logarithmic axis.

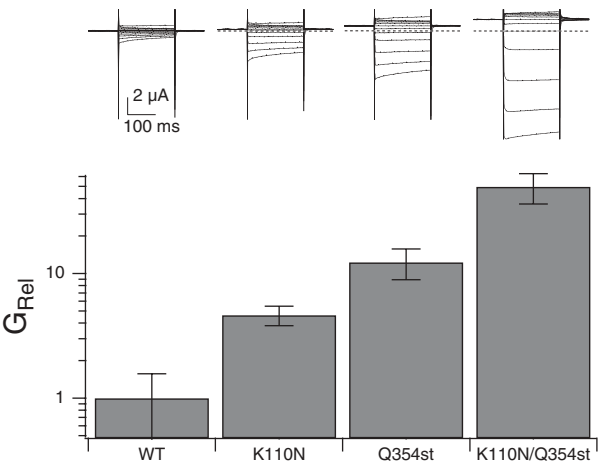

Fig. 4. Double mutation further increases current density. (*Top*) Representative traces show current in 10 mM K^+ without leak subtraction. (*Bottom*) Histogram shows summary data from one set of oocytes. For quantitation, 100 µM Ba^{2+} was used to block Kir current so that data reflect blockable current measured at −140 m*V*. Mutant channel current was statistically greater than wild-type ($P < 0.05$, $n = 4$). Note that the vertical axis is a logarithmic scale.

expressed as a homomultimeric protein in heterologous expression systems (Shuck et al., 1997), but later studies showed that the protein did produce functional currents, although current density was relatively low (Derst et al., 1998; Pearson et al., 1999). It was also shown that mutating a single residue in the first extracellular domain of Kir4.2 (K110N) could dramatically increase the level of current expressed (Derst et al., 1998). Since wild-type Kir4.2 channels have a relatively high P_O (0.5–0.9), the observed (approximately sixfold) increase in current density from the K110N mutation could not be attributed to changes in gating kinetics, and it was concluded that this mutation likely increased the number of channels

reaching the plasma membrane. In the present study, we found that mutations in a specific region of the C terminus of Kir4.2 also increase current density to a similar or greater degree. These mutations either delete or mutate a consensus site for tyrosine phosphorylation by tyrosine kinases such as c-Src (Lin et al., 2002b). Other C-terminal mutations around, but not including, this site did not increase current density. Our findings suggest that tyrosine phosphorylation inhibits Kir4.2 current by reducing the number of active channels in the plasma membrane. When these mutations are combined with the K110N mutation, the increase in current amplitude is greater than the increase of either mutation separately, which suggests that the wild-type channel expression is inhibited by two separate and independent mechanisms.

While these experiments have not addressed the possibility that the observed increase in current density may result from increases in single-channel P_O, it seems an unlikely explanation for several reasons. First, these mutations are in a part of the channel that is not known to be a structural component of the vestibule which surrounds the intracellular portion of the conduction pore. The region of high homology between Kir channels ends more than 15 residues upstream of the Q354st mutation. Second, mutations in more proximal portions of the C terminus associated with the cytoplasmic conduction pore have resulted in *decreases* in P_O. Third, as noted above, several other mutations in similar regions of other Kir channels have been identified as trafficking determinants. Fourth, as the Kir4.2 channel has been reported to have a P_O of ~0.9 (Derst et al., 1998; Pessia et al., 2001), increases in P_O could only account for, at most, a 50% increase in current, whereas we observed increases of 500–1,000%.

Tyrosine phosphorylation is a key regulator of trafficking of the closely related Kir channel Kir1.1 (also called "ROMK"), as shown in an elegant set of studies by Wang, Lin & Sterling (2002). Inhibiting tyrosine phosphorylation increased Kir1.1 current density in oocytes, whereas inhibiting tyrosine phosphatase activity decreased current density in wild-type, but not mutant, channels that lack a phosphorylatable tyrosine at residue 337 (Moral et al., 2001). Furthermore, using green fluorescent protein-tagged Kir1.1 channels, it was shown that the decrease in current density was accompanied by dynamin-dependent channel endocytosis (Sterling et al., 2002). In contrast, phosphorylation of Kir1.1 by protein kinase C (at Ser 4 and 201) increases trafficking to the membrane (Lin et al., 2002a), as does phosphorylation by protein kinase A or SGK-1 on Ser44. These processes appear to be physiologically important for K^+ homeostasis in the distal nephron of the kidney, where density of K^+ channels is related to dietary K^+ intake. In animals on high-K diets, K^+ current density is high, allowing greater K^+ efflux and excretion, while in animals on low-K^+ diets, K channel density is low, helping to conserve serum K^+ (Palmer, Antonian & Frindt, 1994; Wei et al., 2001). While high dietary K does not correspond to increased Kir1.1 RNA transcription (Frindt et al., 1998), low dietary K^+ decreased RNA transcription (Wald et al., 1998). Immunoprecipitation experiments have shown that tyrosine phosphorylation of Kir1.1 is also greater in rats maintained on a low-K diet compared to a high-K diet (Lin et al., 2002b). Since Kir4.2 is also expressed in the kidney, it is conceivable that this newly identified regulatory mechanism may act in concert with regulation of Kir1.1 to modify K^+ homeostasis. Curiously, while Kir1.1 and Kir4.2 are structurally similar, the tyrosine phosphorylation motifs are located at different regions of the C terminus. In Kir1.1, the phosphorylation site is near the end of the extended conserved Kir core domain (at the beginning of βM in the G protein-gated Kir [GIRK] structure [Nishida & MacKinnon, 2002]), while in Kir4.2 the domain we have identified is located in the distal C terminus, in a region that shares strong homology with only Kir4.1. Kir4.1 lacks the phosphorylation site and expresses at a high level (Bond et al., 1994; Shuck et al., 1997).

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