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# Contributions of the C-Terminal Domain to Gating Properties of Inward Rectifier Potassium Channels

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## Summary

**Two inward rectifier potassium channels, the G protein-dependent GIRK1 and the G protein-independent BIR10, display large differences in rectification and macroscopic kinetics. A chimeric channel was constructed in which the putative intracellular carboxy-terminal domain of the G protein-dependent channel replaced the corresponding domain of the G protein-independent channel. The chimeric channel conducted potassium ions without the requirement of activated G proteins, yet displayed activation and deactivation kinetics and rectification properties similar to those of the G protein-dependent channel. The results demonstrate that structural elements in the C-terminus can independently control gating but not G protein signal transduction. The voltage dependence, time course, and kinetics of gating suggest a mechanism in which the pore may be occluded by reversible interactions with charged residues in the C-terminus.**

## Introduction

Inward rectifier potassium channels contribute to the maintenance of resting membrane potential and the control of excitability. They have been found in a wide variety of tissues and cell types, including red blood cells, nerve and glial cells, and cardiac and skeletal muscle cells (Lewis et al., 1991; McKinney and Gallin, 1988; Constanti and Galvan, 1983; Mihara et al., 1987; Barres, 1991; Sakmann and Trube, 1984; Katz, 1949; Standen and Stanfield, 1978). Inward rectifier potassium channels readily permit the inward flow of potassium ions at potentials below the equilibrium potential ($E_K$), but at depolarized potentials outward currents are inhibited. Therefore, in contrast to voltage-dependent potassium channels, inward rectifiers do not activate over a fixed range of voltage; rather, conductance appears to be determined primarily by the driving force on potassium ions. The rectifying nature of the conductance is, in some inward rectifiers, due in large part to a voltage-dependent block of the pore by intracellular cations (Matsuda et al., 1987; Vandenberg, 1987; Lu and MacKinnon, 1994; Wible et al., 1994; Stanfield et al., 1994). However, some inward rectifiers also show an apparent intrinsic gating; the channel open probability increases with hyperpolarization. Interestingly, this process is coupled to the concentration of potassium, indicating an interaction between permeation, block, and gating (Leech and Stanfield, 1981; Hagiwara and Yoshii, 1979; Silver and DeCoursey, 1990). Recent studies suggest a role for cytoplasmic polyamines in the latter process (Lopatin et al., 1994; Fakler et al., 1994).

Some inwardly rectifying potassium channels require the action of activated G proteins. In cardiac atrial cells, parasympathetic stimulation from the vagal nerve activates muscarinic receptors and causes the subsequent activation of an otherwise silent inward rectifier potassium channel, the muscarinic potassium channel; the potassium current results in a slowing of the rate of depolarization and thus of the heart rate (Sakmann et al., 1983; Ijima et al., 1985). The coupling between muscarinic acetylcholine receptor and inward rectifier occurs via a membrane-delimited, pertussis toxin–sensitive pathway (Pfaffinger et al., 1985; for review, see Brown et al., 1991).

Recently, clones encoding members of both of these subtypes of inward rectifiers have been identified, and the channels have been expressed. While many members of the G protein–independent class have already been discovered (Kubo et al., 1993a; Ho et al., 1993; Bond et al., 1994), to date only a single member of the class that requires activated G proteins has been identified, GIRK1 (Kubo et al., 1993b; Dascal et al., 1993). Although they display little sequence conservation with voltage-dependent and calcium- and voltage-dependent potassium channels (Jan and Jan, 1990; Stühmer et al., 1988; Adelman et al., 1992), all of the cloned inward rectifiers share a common architecture thought to consist of two transmembrane domains (TMDs); the amino and carboxyl termini reside within the cell. The TMDs flank the highly conserved P region, which comprises much of the hydrated ion-conducting pore, and within which resides the potassium-selectivity filter (Heginbotham et al., 1992, 1994). Among the cloned inward rectifiers, the degree of rectification varies, and this property is at least in part endowed by the character of a single residue within the second TMD (Stanfield et al., 1994; Lu and MacKinnon, 1994; Wible et al., 1994).

Macroscopic currents in Xenopus laevis oocytes expressing either BIR10, a G protein–independent inward rectifier, or GIRK1, a G protein–dependent channel, display large differences in activation kinetics and degree of rectification. Nevertheless, the channels are structurally related and share 37% amino acid sequence identity. To investigate the structural motifs responsible for imparting these distinct functional characteristics, a chimeric channel was constructed in which the intracellular C-terminus of BIR10 was replaced by the analogous region from GIRK1; these domains share 37% identity. The expressed channel, BG, does not require activated G proteins for gating, but demonstrates the rectification and kinetic properties of GIRK1.

## Results

Expression of BIR10 in Xenopus oocytes results in inward rectifier potassium channels that do not require activated G proteins for gating (Bond et al., 1994). In the presence of

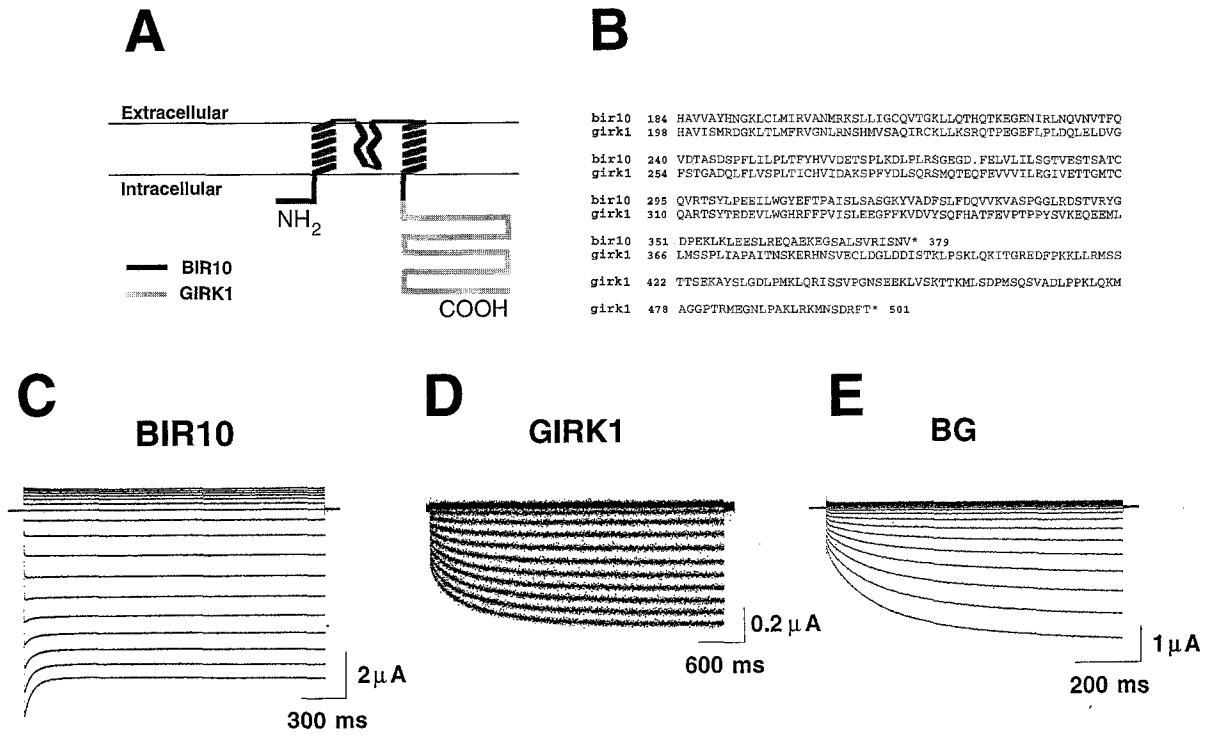


Figure 1. Structure of the BG Chimera and Expression of BIR10, GIRK1, and BG in Xenopus Oocytes

(A) Schematic representation of the BG chimera. The N-terminus, first TMD, pore region, and second TMD are donated by BIR10, while the C-terminal domain is donated by GIRK1.

(B) The amino acid sequences of the exchanged domain; gaps were introduced to optimize the alignment.

(C–E) Current families from oocytes expressing BIR10 (C), GIRK1 (D), and BG (E). Current traces were recorded in 90 mM external potassium and were evoked by voltage commands from a holding potential of −10 mV. Commands were delivered in −10 mV increments from +50 to −110 mV. (D) shows a current family evoked from an oocyte coinjected with dopamine D2 receptor and GIRK1 mRNA following application of 60 μM dopamine. Records were obtained by subtracting traces from the same oocyte before and after application of dopamine. Addition of 60 μM dopamine to either oocytes expressing BG alone or oocytes expressing BG with the dopamine D2 receptor had no effect on the currents (data not shown).

90 mM external potassium, hyperpolarizing voltage pulses from a holding potential of −10 mV evoked BIR10 currents that develop to a peak amplitude within several milliseconds (Figure 1C). Increasing hyperpolarization results in relaxation of the peak current to a reduced steady-state amplitude. To study GIRK1, which requires G protein activation (Kubo et al., 1993b), channels were coexpressed with dopamine D2 receptors (Bunzow et al., 1988). Current records obtained during a series of voltage pulses in control solution were subtracted from the current records obtained during application of 60 μM dopamine (Figure 1D); application of dopamine to oocytes injected only with dopamine D2 receptors had no effect (data not shown). Expression of GIRK1 resulted in inward rectifier potassium currents that activate much more slowly than BIR10 currents following membrane hyperpolarization (Figure 1). In addition, voltage pulses to potentials positive to $E_K$ demonstrate that GIRK1 rectifies more strongly than BIR10 (Figures 1C and 1D; Figure 2). Application of dopamine to oocytes coexpressing BIR10 and dopamine D2 receptors had no effect on BIR10 currents (data not shown).

To investigate the structural components underlying the differences in G protein dependence, rectification, and activation kinetics, chimeric channel subunits were constructed in which the putative intracellular C-terminal domains from GIRK1 and BIR10 were exchanged (amino acid residues 184–379 in BIR10 and 198–501 in GIRK1; Figure 1B). The chimeric channel GB, in which the N-terminal domain, first TMD, pore, and second TMD were donated by GIRK1 and in which the C-terminal domain was donated by BIR10, did not mediate potassium currents when expressed in Xenopus oocytes, either alone or in concert with dopamine D2 receptors and application of dopamine. In contrast, the chimeric channel BG, in which the N-terminal domain, first TMD, pore, and second TMD were donated by BIR10 and in which the C-terminal domain was donated by GIRK1, did produce functional channels (Figure 1A). These channels did not require activated G proteins; coexpression with dopamine D2 receptors and application of dopamine did not alter the current amplitudes (data not shown). BG channels displayed kinetics similar to those of GIRK1, and the current relaxation seen with BIR10 channels at more hyperpolarized potentials was absent (Figure 1E). The potassium current–voltage relationships reveal that, at potentials more positive than $E_K$, the chimeric channel demonstrates rectification prop-

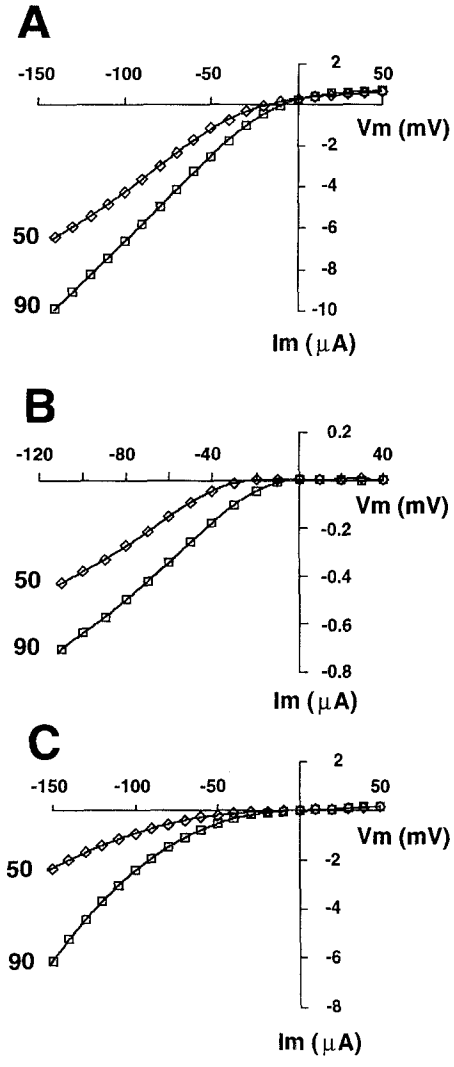


Figure 2. Current–Voltage Relationships for BIR10 Peak Currents and GIRK1 and BG Steady-State Currents

The current–voltage relationships for BIR10 (A), GIRK1 (B), and BG (C) currents were determined in either 50 or 90 mM external potassium.

erties similar to those of GIRK1; outward currents are significantly more reduced than for BIR10 (Figure 2). At potentials negative to $E_K$, the inward current–voltage relationship of the chimera reflects alterations in the gating process (see below).

Potassium selectivity (Bond et al., 1994; Kubo et al., 1993b) and sensitivity to block by cesium and barium (Bond et al., 1994) were tested in the BG chimeric channel. As shown in Figure 3A, as the external potassium concentration was altered, the reversal potential shifted in accord with the Nernst equation for a potassium-selective conductance (potassium substituted by either NaCl [n = 6] or N-methyl-D-glucamine [n = 8]). Application of external barium or cesium to oocytes expressing BG produced a concentration- and voltage-dependent block of the channels (Figure 3B). The apparent fraction of the membrane electric field sensed by the blocking ion was calculated to be 0.48 ± 0.02 (n = 7) for barium and 1.95 ± 0.12 (n = 6) for cesium (Woodhull, 1973). These values are similar to those previously determined for BIR10 (Bond et al., 1994), which contributes the structural domain encompassing the P region of the BG chimera. However, the zero voltage $K_D$ values were also determined (99.3 ± 2.3 μM [BIR10] and 210 ± 26 μM [BG] for barium block; 112 ± 17 mM [BIR10] and 210 ± 75 mM [BG] for cesium block) and yielded values that are significantly different for BIR10 and BG. This may reflect C-terminal structural contributions to the inner portion of the conduction pathway, as has been previously suggested (Taglialatela et al., 1994).

The kinetics of channel activation were compared between GIRK1 and BG. In both cases, the time dependence of the current following a hyperpolarizing pulse was well fitted by the sum of two exponential functions (GIRK1, Figures 4A and 4B; BG, Figures 4C and 4D). The fast and slow time constants were very similar for the two channels and displayed similar voltage dependencies. In both cases, the slow time constant displayed a bell-shaped dependence on the membrane potential (Figures 4B and 4D). The time course of BG activation was also examined using either 10 or 90 mM external potassium. When commands to −120 mV were delivered from a holding potential of −10

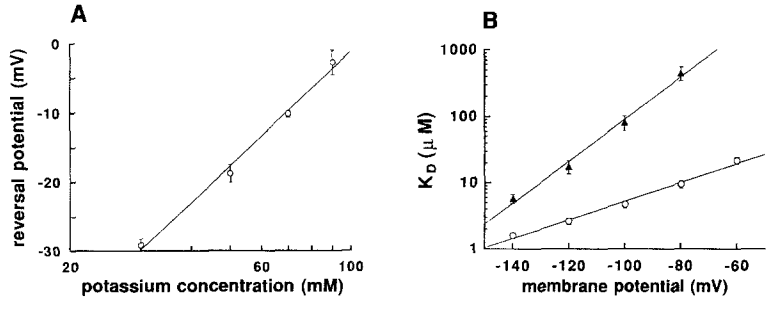


Figure 3. Pore Properties of BG

(A) BG is a potassium-selective channel. The reversal potential is plotted as a function of the external potassium concentration (30, 50, 70, and 90 mM, replaced by NaCl; n = 6). The straight line shows the equilibrium potential for potassium predicted by the Nernst equation. Vertical bars indicate SEM.

(B) Voltage dependence of external barium (circles) and cesium (triangles) block for BG steady-state currents. Concentration–response experiments were performed in 90 mM external potassium by delivering a 2 s voltage command from a holding potential of −10 mV to the indicated potentials. The $K_D$ values were determined by fitting the data to a logisitic equation (see Experimental Procedures) and were plotted as a function of the command potential. The Hill coefficient was not different from 1. These data were fitted by the Woodhull equation (Woodhull, 1973) $K_D = K_D(0\ mV)\exp(\delta zFE/RT)$, and the δ value was determined. Vertical bars indicate SEM.

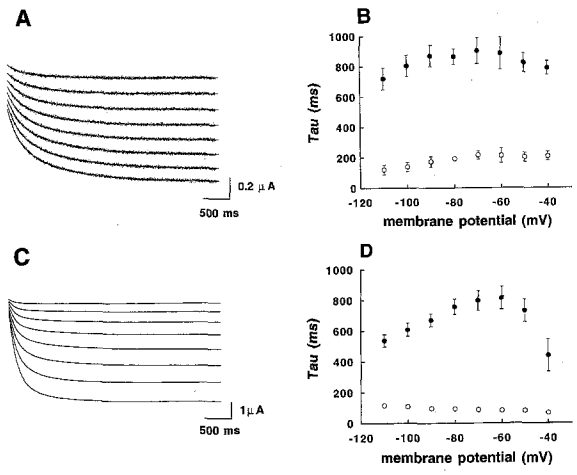


Figure 4. Kinetics of Activation for GIRK1 and BG

Representative current families for GIRK1 (A) and BG (C) recorded in 90 mM external potassium. Voltage commands of 4 s were delivered from a holding potential of −10 mV to command potentials from −40 mV to −110 mV in −10 mV increments. The current traces shown have been fitted with the sum of two exponential functions. Fast (open circles) and slow (closed circles) time constants for GIRK1 (B) and BG (D) are plotted as a function of command potential.

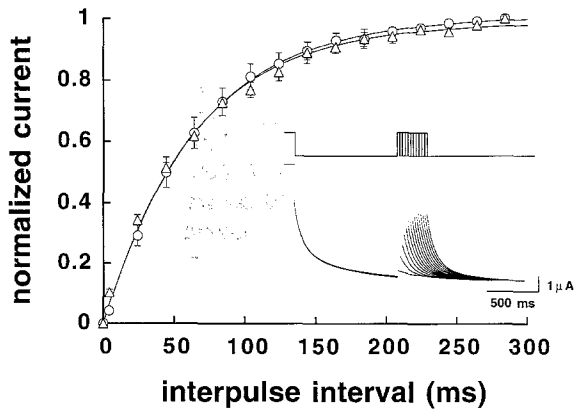


Figure 5. Time Course of Deactivation

A two-pulse protocol (inset) was applied to oocytes expressing GIRK1 (triangles) or BG (circles). Hyperpolarizing commands (1 s) to −100 mV delivered from a holding potential of −10 mV were followed by a depolarizing command to −10 mV for a variable period of time. A second hyperpolarizing voltage pulse to −100 mV was then delivered, and the normalized amplitude of the time-dependent currents was plotted as a function of the time interval at −10 mV. Time constants were derived by fitting the data points with a single exponential function.

mV, both the fast and slow time constants were decreased in higher external potassium (10 mM: $\tau_f$ = 134 ms, $\tau_s$ = 1432 ms; 90 mM: $\tau_f$ = 102 ms, $\tau_s$ = 625 ms; n = 3). Similar results were obtained when the holding potential was set at $E_K$ (−10 mV for 90 mM $[K^+]_o$, −60 mV for 10 mM $[K^+]_o$).

The time course of deactivation of GIRK1 and BG following depolarization to −10 mV was examined using a paired-pulse protocol. Channels were activated by a 1 s hyperpolarizing command pulse to −100 mV from a holding potential of −10 mV, and time-incrementing depolarizing command pulses to −10 mV were then applied, followed by a second pulse to −100 mV (Figure 5, inset). The current trace from the second hyperpolarizing command was fitted by the sum of two exponential functions, and the instantaneous current was determined by extrapolation to time zero. This value is proportional to the number of open channels at the end of the depolarizing command (Silver and DeCoursey, 1990). The plot of the normalized, time-dependent current ($I_{inst}$–$I_{ss}$) as a function of the interpulse interval was well fitted by a single exponential function, yielding time constants of 63.2 ± 2.3 ms for GIRK1 (n = 3) and 65.8 ± 1.0 ms for BG (n = 3; Figure 5).

For GIRK1 and BG, the voltage dependence of the gating process was examined using a protocol in which the prepulse potential was varied, followed by a step to −110 mV (Figure 6A). The instantaneous change in current following the hyperpolarizing voltage jump was determined by extrapolation of the fitted currents to time zero, and used to calculate the instantaneous conductance. The conductance–voltage relationships were well fitted by Boltzmann functions, and the apparent valence of the charge movement underlying the gating process was similar for the two channels (1.4 for GIRK1 and 1.1 for BG; n = 3). The voltage midpoint of the conductance activation was −17 mV for GIRK1 and −60.4 mV for BG (n = 3; Figure 6B).

## Discussion

BIR10 and GIRK1 are members of the inward rectifier potassium channel family. They share a common predicted membrane topology and are 37% identical in primary sequence. Although both channels are potassium-selective inward rectifiers, they exhibit several major functional differences: GIRK1 requires activated G proteins for gating, whereas BIR10 does not; the voltage-dependent activation kinetics are significantly faster for BIR10 than for GIRK1; BIR10 displays a voltage-dependent current decay at hyperpolarized potentials that is absent from GIRK1 currents; and the GIRK1-mediated potassium conductance is more strongly rectifying than that of BIR10.

When the intracellular C-terminal region of GIRK1 was substituted for the corresponding region in BIR10, the chimeric channel BG retained the essential pore properties of potassium selectivity and block by barium and cesium. Channels were activated by hyperpolarization, independently of the actions of G proteins. However, the activation and deactivation kinetics and the rectification properties of the BG chimera were indistinguishable from those of GIRK1. In the BG channel, the voltage-dependent relaxation of the current at more hyperpolarized potentials associated with BIR10 and the requirement for the actions of activated G proteins for GIRK1 are absent.

The data presented here differ from those presented by Takao et al. (1994), who examined an analogous chimera in which the C-terminal domain of GIRK1 was substituted for the corresponding region of the inward rectifier IRK1 (Kubo et al., 1993a). The discrepancies may be due

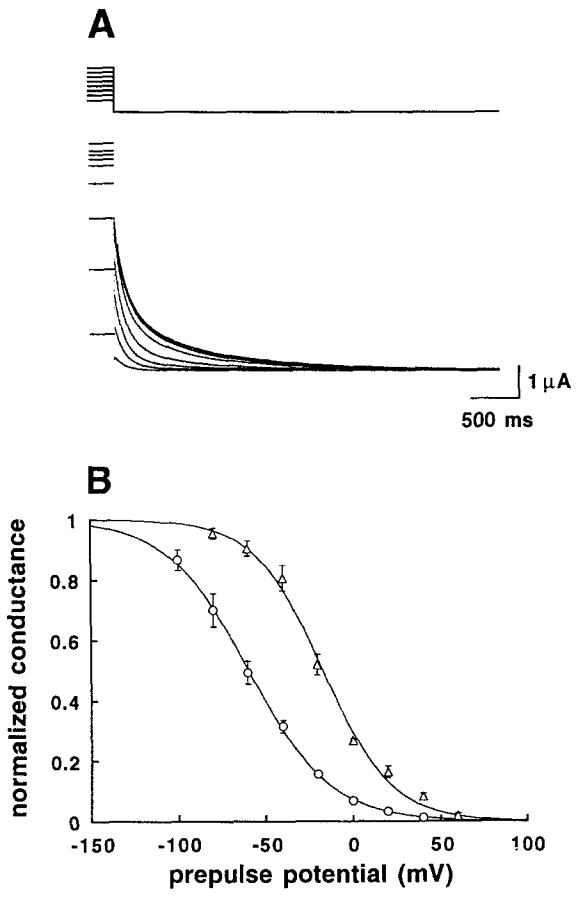


Figure 6. Voltage Dependence of Gating

The prepulse potential was varied from +60 mV to −80 mV (GIRK1) or −100 mV (BG), followed by a hyperpolarizing command to −110 mV (A). The instantaneous change in current was determined by extrapolation of fits of current traces to the sum of two exponential functions to time zero; from this value, the instantaneous conductance was derived and plotted as a function of the prepulse potential (B). The data were fitted to $g/g_{max} = 1/\{1 + \exp[zF(V-V_{½})/RT]\}$. Triangles, GIRK1; circles, BG.

to the slightly different region of GIRK1 employed for chimera construction, as Takao et al. found that acetylcholine enhancement of currents was transferred when amino acids 339–501 of GIRK1 were substituted after amino acid 360 of IRK (IGC2). However, BG contains more of the C-terminal domain of GIRK1 than IGC2 (residues 198–501). Alternatively, the different results may be due to specific structure–function differences between the parent inward rectifiers, IRK1 and BIR10. Takao et al. reported enhancement of current amplitudes when acetylcholine was applied to oocytes expressing IRK1 and mAChR2, while BIR10 currents are not altered under similar conditions (data not shown). Further chimeric subunits presently under investigation may provide a more reasonable understanding of these differences.

The data presented here are consistent with those reported by Taglialatela et al. (1994), who found that exchange of the C-terminal domains between IRK1 and ROMK1, two G protein–independent inward rectifiers, transferred rectification properties even though the rectification residue in the second TMD was not exchanged. Taglialatela et al. also concluded that the C-terminal domain contributes to conduction properties, consistent with the results presented here for cation block of BIR10 and BG. Together, these results support the conclusion that the C-terminal domain contains structural information for gating and conduction properties of inward rectifier potassium channels.

Recent data demonstrate that the influence of activated G proteins is transduced to the GIRK1 channel by the βγ subunits (Reuveny et al., 1994), and in native tissues such as cardiac myocytes, this process occurs in a membrane-delimited fashion (Pfaffinger et al., 1985; Brown et al., 1991). The slow kinetics of GIRK1 activation might be due to the time for dissociation of the heterotrimeric G protein complex, to diffusion in the membrane, and to either direct or indirect allosteric effects on the channel that initiate the gating process. In contrast, cloned inward rectifiers such as BIR10 activate much more rapidly. The results presented here suggest that these differences are not due to the influence of G protein subunits on GIRK1, but reflect fundamental differences in the intrinsic mechanisms of channel gating.

Inward rectification is due, at least in part, to a voltage-dependent blockade of the channel pore by intracellular cations such as magnesium ions and polyamines (Matsuda et al., 1987; Vandenberg, 1987; Lopatin et al., 1994; Fakler et al., 1994). Recently, a single amino acid residue in the second TMD of cloned inward rectifiers has been identified that influences the degree of rectification, presumably through an electrostatic effect on the blocking ion. Among the cloned inward rectifiers, this residue is usually either asparagine, resulting in weaker rectification, or aspartate, resulting in stronger rectification (Stanfield et al., 1994; Lu and MacKinnon, 1994; Wible et al., 1994; Fakler et al., 1994). In the case of BIR10, the amino acid corresponding to this "rectification residue" is glutamate, and in GIRK1 it is aspartate. Although both are negatively charged, there is significantly stronger rectification of the GIRK1 currents (Bond et al., 1994; Kubo et al., 1993b) (see Figure 1 and Figure 2). Moreover, in the BG chimera, this position is donated by BIR10, yet the chimeric channel has rectification properties indistinguishable from those of GIRK1, which suggests an additional structural determinant for cation binding or intrinsic gating localized to the C-terminal domain. From the voltage dependence of activation of this gating process, the product of the charge on the residues and the fraction of the field that they traverse during the closed-to-open channel transition (e.g., zδ value) is predicted to be 1.1–1.4. This value is consistent with binding of a polyvalent amine at a site traversing a fraction of the membrane electric field. A second mechanism consistent with these data involves the reversible interaction of charged residues in the C-terminal domain with a site in the membrane electric field, which would cause occlusion of the channel pore. Indeed, there are several islands of charged residues within the C-terminal domain of GIRK1 that might function as the blocking domain. In this regard, it is interesting that both time constants of activation for BG are faster when the external

solution contains higher concentrations of potassium. Thus, inward rectifier gating may involve a fast block by intracellular cations, a slow block by a domain of the channel protein, and interactions betweeen these processes and permeant ions. Further mapping of the C-terminal domain of GIRK1 using chimeras and site-directed mutations will likely identify the residues that underlie this gating process.

## Experimental Procedures

### Molecular Biology

GIRK1 (KGA) was the generous gift of Drs. H. Lester and N. Dascal, and the D2 receptor was kindly provided by James Bunzow. BG was constructed by digesting GIRK1 and BIR10 at a shared SphI restriction site. Appropriate restriction fragments were isolated from agarose gels and purified by electroelution. Following ligation into pSK (Stratgene) and transformation of E. coli DH5α bacteria, the nucleotide sequence of BG was verified using Sequenase T7 DNA polymerase (US Biochemicals). Enzymes were purchased from BRL.

### Oocyte Expression and Electrophysiology

In vitro synthesis of mRNA and oocyte injection and handling have been described (Christie et al., 1989). Two-electrode voltage clamp recordings were made with a Geneclamp 500 amplifier (Axon Instruments) interfaced to a Macintosh Quadra 800 computer using an ITC16 A/D board (Instrutech), with either Pulse or Axodata software. Recordings were performed 2–10 days after RNA injection. Oocytes were continuously superfused with a solution containing 90 mM KCl, 3 mM $MgCl_2$, and 10 mM HEPES (pH 7.4) at room temperature (21°C–23°C). For experiments with different external potassium solutions, potassium was substituted by NaCl or N-methyl-D-glucamine. Concentration–response data for barium and cesium inhibition were fitted by least squares to the function $i = [ion]^n/(K_D^n + [ion]^n)$, where i was the fractional inhibition of the steady-state current resulting from barium or cesium superfusion. Data analysis was performed using Axograph, Pulse Fit, and Kaleidograph software.

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