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Functional Expression and Characterization of G-protein-gated Inwardly Rectifying K^+ Channels Containing GIRK3

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Abstract. The G-protein-gated inwardly rectifying K^+(GIRK) family of ion channels form functional Gβγ-sensitive channels as heteromultimers of GIRK1 and either the GIRK2 or GIRK4 subunits. However, the homologous mouse brain GIRK3 clone failed to express in the earliest reported functional experiments in *Xenopus* oocytes. We recloned the GIRK3 subunit from mouse brain and found that the new clone differed significantly from that originally reported. The functional aspects of GIRK3 were reinvestigated by expression in CHO cells. The single channel properties of GIRK1/GIRK3 were characterized and compared to those of the GIRK1/GIRK2 and GIRK1/GIRK4 channels. All three GIRK1/GIRKx combinations produced channels with nearly indistinguishable conductances and kinetics. The response of GIRK1/GIRK3 to Gβγ in the 1–47 nM range was examined and found to be indistinguishable from that of GIRK1/GIRK4 channels. We conclude that GIRK1, with either GIRK2, 3, or 4, gives rise to heteromultimeric channels with virtually identical conductances, kinetics, and Gβγ sensitivities.

Key words: Potassium channels — Kir 3.3 — Gβγ—Ion channels

Introduction

The inward rectifier K^+ channels are encoded by a superfamily of genes that includes seven currently recognized families (Kir 1.0–7.0). The Kir channel structure consists of two transmembrane domains, a K^+-selective pore region, and intracellular amino and carboxy termini. GIRK3 is a member of the Kir 3.0 family of G-protein-regulated inwardly rectifying K^+ channels which contains four mammalian subunit members. Overall the four mammalian GIRK subunits share 36% identity, but in the pore and transmembrane regions the level of identity is 80–90%. GIRK2, GIRK3 and GIRK4 are the most closely related, sharing 62% identity overall. GIRK1, the largest subunit, is also the most divergent of the four, sharing only 44% identity with the other three.

The cardiac inward rectifier I_{KACh} is a tetramer composed of GIRK1 and GIRK4 subunits (Krapivinsky et al., 1995*b*; Corey et al., 1998). GIRK1 and GIRK2 have also been co-immunoprecipitated from rat cerebral cortex, hippocampus, and cerebellum (Liao, Jan & Jan, 1996), and GIRK1, 2 and 3 subunits are widely distributed in brain (Karschin et al., 1994; Kobayashi et al., 1995 Chen et al., 1997; Karschin & Karschin, 1997). In functional studies in *Xenopus* oocytes, injection of a GIRK1/GIRKx combination of subunits yields significantly more current than any GIRK subunit injected alone or any other combination of GIRK2, 3, or 4 subunits (Duprat et al., 1995; Kofuji, Davidson & Lester, 1995; Kraivinsky et al., 1995*b*; Dissmann et al., 1996; Velimirovic et al., 1996; Wischmeyer et al., 1997). GIRK1 participation appears to be key for the appearance of normal kinetic properties of the heteromultimer, but it is not necessary for Gβγ gating (Krapvinsky et al., 1998).

After the GIRK3 clone originally reported failed to express with GIRK1, whole cell currents of 3 μA at −80 mV were demonstrated in *Xenopus* oocytes injected with GIRK1/GIRK3 (Kofuji et al., 1995; Dissmann et al., 1996; Wischmeyer et al., 1997). However, compared to studies focused on GIRK1/GIRK2 and GIRK1/GIRK4 little work has focused on characterizing the GIRK1/GIRK3 current. This is unfortunate since GIRK3 may be

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the most abundant of brain GIRK2, 3, or 4 subtypes (Kobayashi et al., 1995; Karschin et al., 1996; Chen et al., 1997; Karschin & Karschin, 1997). We found that the recloned GIRK3 was significantly different from the previously reported mouse sequence (Lesage et al., 1994), but was almost identical to clones from other species. We expressed the new mouse brain GIRK3 and GIRK1 and compared it to GIRK1/GIRK2 and GIRK1/GIRK4 expressed in the same mammalian cell line. The present study shows that GIRK3, when coexpressed with GIRK1 in CHO cells, produces an inwardly rectifying K^+ current similar to that seen with the GIRK1/GIRK2 and GIRK1/GIRK4 channels. These results reinforce the notion that GIRK1 combines with either GIRK2, 3, or 4 subunits to produce the typical mammalian GIRK channel phenotype and that all GIRK1/GIRKx heteromultimers have similar gating and conductance properties.

Materials and Methods

Cloning

Total poly-A RNA was isolated from adult mouse brain using the Poly (A) Pure Kit (Ambion, Austin, TX). Five separate reverse transcription reactions were run, each using 0.5–1.5 μg poly-A RNA as template (Pharmacia Biotech, Piscataway, NJ). PCR primers were designed based on the published 5′ UTR and 3′ UTR sequences for mouse brain GIRK3 (Lesage et al., 1994). The forward primer (5′-GCTCTAGAGGGAACCTAGGGTACTGGGG) introduced a Xba1 restriction site into the 5′ end. The reverse primer (5′-GCGAATTCACCAGTCACACCTTGGACTCAC) introduced an EcoR1 site into the 3′ end. Five separate PCR reactions were run using the five RT products as templates using Vent DNA Polymerase (New England Biolabs, Beverly, MA).

The PCR reaction was carried out for 40 cycles under high stringency conditions (98°C, 60 sec; 65°C, 90 sec; 72°C, 120 sec). All five PCR reactions produced a single band product of the expected size (~1.4kb). Three PCR products were pooled, double digested with Xba1 and EcoR1 (Promega, Madison, WI), and subcloned into the pcDNA3.1(−) vector (Invitrogen, Carlsbad, CA) using the Ligation Express Kit (CLONTECH Laboratories, Palo Alto, CA). The other two PCR products were incubated with Taq DNA Polymerase (Boehringher Mannheim, Indianapolis, IN) at 72°C for 10 min in the Perkin Elmer GeneAmp 2400 and then subcloned into the pCRII vector using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). At least two positive colonies per transformation were sequenced completely on both strands.

Xenopus Oocyte Recording

Stage V and VI *Xenopus* oocytes were defolliculated by collagenase treatment the day before injection. Oocytes were injected with 1.2 ng of each GIRK subunit and 0.6 ng each of $G\beta_1$ and $G\gamma_2$ mRNAs. mRNAs were in vitro transcribed from cDNA clones using the mMessage mMachine kit (Ambion, Austin, TX). Oocytes were assayed for whole cell currents by two-electrode voltage clamp (NPI Turbotec, Darmstadt) and filtered at 1 kHz (8-pole Bessel filter) two days post injection. The bath solution consisted of (in mM) 96 KCl, 2 NaCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 Na-HEPES (pH 7.4).

Channel Expression

Chinese hamster ovary cells (CHO-K1, ATCC accession No. CCL-61) were chosen for heterologous expression of GIRK channels. The cells were grown and maintained in 1:1 DMEM:Hamm's F-12 (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (Sigma Chemical, St. Louis, MO) in a tissue culture incubator at 37°C under 5% CO_2. CHO-K1 cells were plated one or two days before transfection to ~50% confluency at the time of transfection. Cells were transfected in OptiMEM I media (Gibco BRL, Gaithersburg, MD) overnight using *Trans*IT™-Lt1 (Pan Vera, Madison, WI). Green flourescent protein (Plasmid pGreen Lantern, Gibco BRL, Gaithersburg, MD) was cotransfected with the GIRKs in a 1:4 ratio as a transfection marker, while different GIRKs were transfected in a 1:1 ratio. A total of 5 μg of DNA was transfected per 60 mm^2 dish. Transfected cells were detached with 5 mM EDTA in PBS (in mM): 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, 12 Na_2PO_4 resuspended in CHO growth media, and transferred to glass coverslips for patch-clamp experiments.

Patch-Clamp Recording

Patch electrodes were prepared from borosilicate capillary glass (Warner Instrument, Hamden, CT) and polished to a resistance of 5–12 MΩ. A high K^+ solution consisting of (in mM); 140 KCl, 2 $MgCl_2$, 5 EGTA, and 10 HEPES, pH 7.2 was used as the standard bath and electrode solution. Patches were studied in the inside-out configuration with either GTPγS (Boehringher Mannheim, Indianapolis, IN) or purified bovine brain Gβγ (Krapivinsky et al., 1995*b*) added by pipette to the bath solution. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered at 2kHz by a 4-pole low-pass Bessel filter and sampled at 10 kHz.

Records of channels in inside-out patches activated by GTPγS were collected at −80, −70, −60, −50, and −40 mV. All-points-histograms were made using pCLAMP6's Fetchan module (Axon Instruments, Foster City, CA). Gaussian curves were fit to the data using Origin 5's Professional module (Microcal Software, Northampton, MA). Origin's pCLAMP6 module was used to make all-points-histograms for records with low signal-to-noise ratios. Unitary current values from all patches were averaged and the slope conductance was determined from the best fit to the mean data. Channel open times were determined for 10 sec records of inside-out patches activated by GTPγS collected at −80 mV transmembrane voltage. Log analysis (Sigworth & Sine, 1987) was used for determination of channel mean open time.

Purified bovine brain Gβγ (Krapivinsky et al., 1995*b*) activated inside-out patches containing GIRK1/GIRK4 and GIRK1/GIRK3 channels. Patches were excised into the standard bath solution and basal activity was recorded for 2.5 min before Gβγ was added to an initial concentration of 1 nM. Additional Gβγ was added incrementally at 2.5-min intervals until a final concentration of 17 nM or 47 nM was reached. 2.5 minutes after the highest Gβγ concentration was attained, 100 nM GTPγS was added to the bath. For the final minute of each Gβγ concentration step, the product NP_o (where N = the number of channels in the patch and P_o is the open probability of the channel) was measured. NP_o values were normalized to the final activity level 1.5 min after the addition of GTPγS for each patch in order to derive relative NP_o values. Relative NP_o values from all patches were aver-

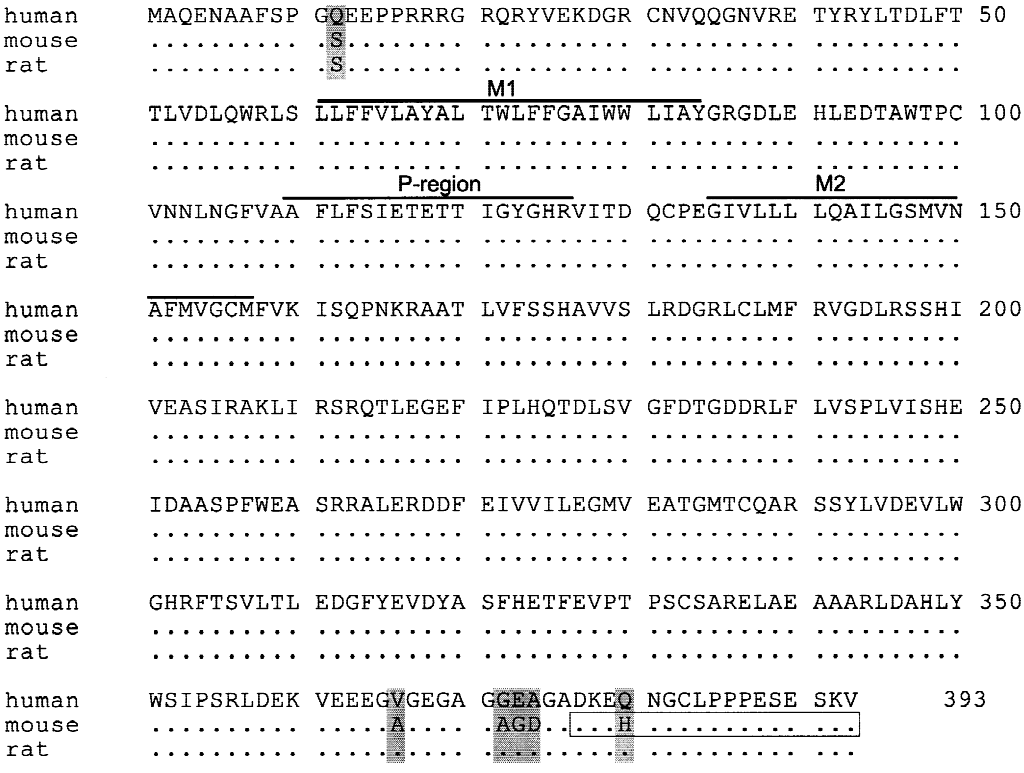

Fig. 1. The deduced amino acid sequences for GIRK3 clones from human, mouse, and rat are shown. The mouse sequence is in GenBank under accession number AF130860. The human sequence was submitted to GenBank by Schoots and Van Tol under accession number U52152 and the rat brain sequence was submitted to GenBank by Dissmann et al. under accession number L77929. Consensus is indicated by dots and divergent residues are shaded. The transmembrane regions M1 and M2 and the pore region are overlined and the seventeen amino acid addition is boxed.

aged and the dose-response curve was determined as the best fit to the mean data.

Results

Cloning of GIRK3

The cDNA of GIRK3 was cloned by RT-PCR from adult mouse brain. The amino acid sequence deduced from our cDNA sequence differed from the previously reported mouse brain sequence (Lesage et al., 1994) by two amino acids near the first transmembrane region (M1; serine instead of arginine at residue 60 and alanine instead of valine at residue 77) and by a seventeen amino acid addition at the carboxy terminus. The reported human and rat amino acid sequence agreed with our sequence (Fig. 1). Even with the additional length reported here, GIRK3 remains the smallest of the four mammalian GIRK subunits, with 393 amino acids, while GIRK1 is the largest subunit, containing 501 amino acids. The longest splice variant of GIRK2 contains 423 amino acids and GIRK4 contains 419 amino acids.

Characterization of GIRK1/GIRK3 Single Channels in CHO Cells

The new GIRK3 clone was expressed first in *Xenopus* oocytes and the ionic currents assayed. The currents recorded had kinetics and amplitudes that were comparable to those seen with the GIRK1/GIRK2 channels, reaching a maximal current amplitude of 8 μamps at −80 mV (*see* Velimirovic et al., 1996).

The single-channel properties of GIRK1/GIRK3 channels were investigated by transient transfection in CHO cells and compared with the single channel characteristics of GIRK1/GIRK2 and GIRK1/GIRK4 in the same cell line. Only cells exhibiting GFP fluorescence were assayed by patch clamp. A ramp protocol from +50 to −150 mV transmembrane voltage was used to verify inward rectification. Patches exhibiting inwardly rectifying channel activity were recorded at −80, −70, −60, −50, and −40 mV. Unitary current values from all patches were averaged and the slope conductance was determined from the best fit of the mean data. GIRK1/GIRK3 channels inwardly rectified and had a slope conductance of 39 ± 2.3 pS (±SEM; $n = 14$; Fig. 2).

In agreement with the results of previous studies,

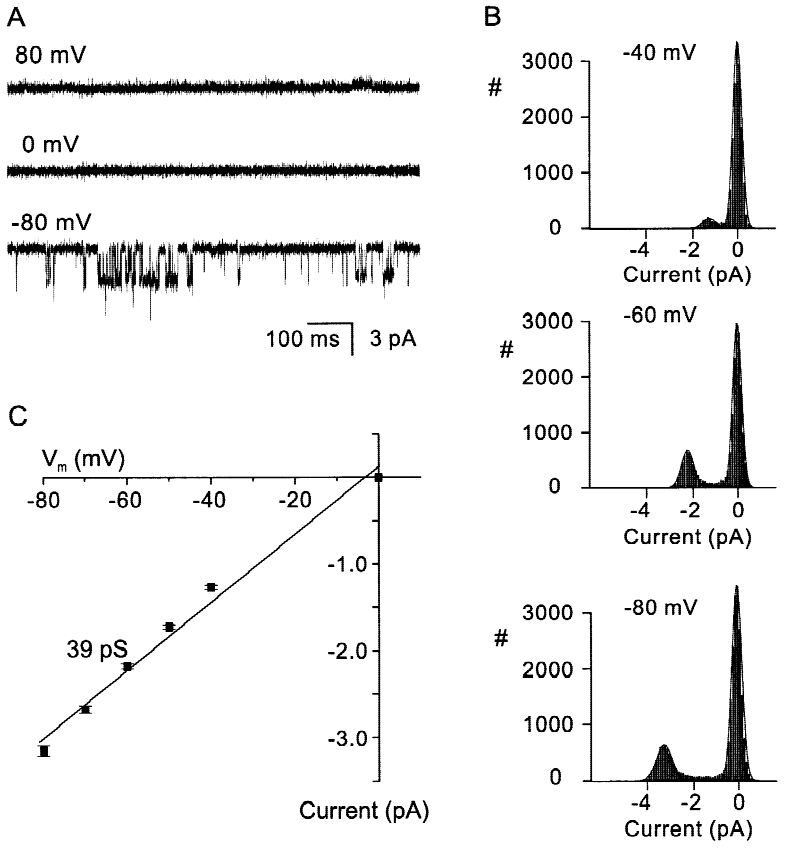

Fig. 2. Single channel properties of GIRK1/GIRK3 expressed in CHO cells. (*A*) Channel currents were recorded at various potentials in the inside-out patch-clamp configuration. K^+ was symmetrical, making the reversal potential 0 mV. Channels were activated with GTPγS (10 μM) in the bath solution. (*B*) All-points-histograms for voltages shown. (*C*) Current-voltage relation reveals a unitary conductance of 39 pS. Data points are given as mean ± SEM, $n = 14$ patches.

GIRK1/GIRK2 and GIRK1/GIRK4 single channel conductances were 35 ± 3.0 pS (±SEM; $n = 12$) and 37 ± 2.5 pS (±SEM; $n = 12$), respectively. Thus GIRK1/GIRK2, GIRK1/GIRK3, and GIRK1/GIRK4 conductance levels were indistinguishable. GIRK1/GIRK3 channel mean open time (τ_o) at −80 mV was 1.4 ± 0.1 msec (±SEM; $n = 7$). The average τ_o for GIRK1/GIRK4 was 1.3 ± 0.1 msec (±SEM/ $n = 5$) and for GIRK1/GIRK2 was 1.2 ± 0.1 msec (±SEM; $n = 11$). We do not consider the differences among the three values for τ_o to be significant. In conclusion, GIRK1/GIRKx combinations produce channels with similar single channel conductance and mean open time (Fig. 3).

GIRK1/GIRK3 Gβγ Sensitivity

Since activation by Gβγ is the hallmark feature of GIRK channels, Gβγ sensitivity was examined for GIRK1/GIRK3 channels by exposing the cytoplasmic faces of inside-out patches to increasing concentrations of Gβγ and monitoring the change in channel activity. Both GIRK1/GIRK3 and GIRK1/GIRK4 showed some basal channel activity in the absence of Gβγ or GTPγS and the activity of both channels increased in response to increasing Gβγ in the concentration range tested (Fig. 4). Sigmoidal fits were made to the mean data for both channels and a half-maximal Gβγ concentration for the range tested was deduced from the fitted curve. The half-maximal Gβγ concentration for GIRK1/GIRK3 was 11 ± 4 nM (compared to 10 ± 3 nM for GIRK1/GIRK4). The half-maximal concentration result is close to the 11.0 nM half-maximal concentration value for $dG\beta_1\gamma_2$ for native GIRK1/GIRK4 in atrial myocytes (Wickman et al., 1994). The half-maximal concentrations for this range are similar for GIRK1/GIRK4 and GIRK1/GIRK3 and both curves were fit by a Hill coefficient of 1.5, similar to the Hill coefficient of 1.7 reported for recombinant $G\beta_1\gamma_2$ for native GIRK1/GIRK4 in atrial myocytes (Krapivinsky et al., 1995*c*). Within the Gβγ concentration range tested, the GIRK1/GIRKx channels have similar sensitivities to Gβγ.

Discussion

We generated a mouse brain GIRK3 clone that differed somewhat from the amino acid sequence previously published for mouse brain GIRK3 (Lesage et al., 1994), but was nearly identical to GIRK3 sequences reported for other species. Interestingly, the sequence contained an

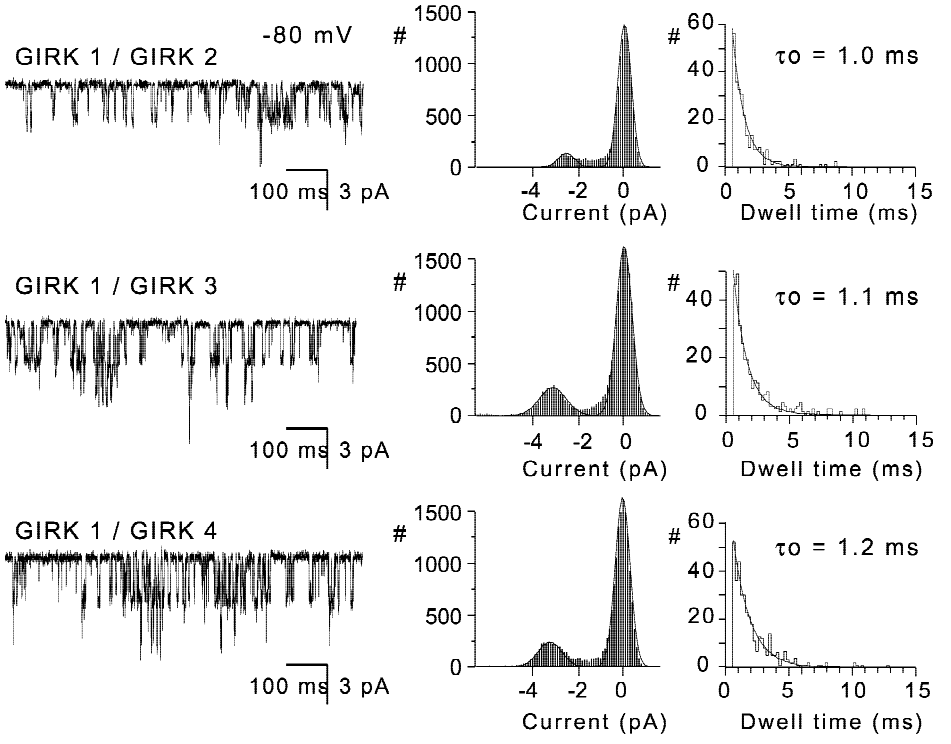

Fig. 3. Heteromultimeric GIRK channels consisting of GIRK1 in combination with GIRK2, GIRK3, or GIRK4 expressed in CHO cells show similar single channel properties with respect to conductance and τ_o. The left panel shows channel activity at −80 mV. The center panel shows the unitary current at −80 mV as measured from the all-points-histogram. The right panel shows the open time distribution at −80 mV as measured from dwell time histograms.

extra seventeen amino acids at the carboxy terminal which ends in −ESKV, the proposed binding motif for the second PDZ domain of PSD-95, -E-S/T-X-V (Sheng, 1996; Craven & Bredt, 1998). The same carboxy terminal motif is seen in the longest splice variant of GIRK2 but not in GIRK1 or GIRK4. This may indicate a role for cytoskeletal proteins like PSD-95 in the subcellular localization of channels containing GIRK2 or GIRK3, as reported for another type of inward rectifier K^+ channel, Kir 4.1 (Horio et al., 1997). It is interesting to speculate that the diversity of GIRK subunits may be related to cell or tissue localization since the biophysical properties of the channels and their sensitivity to Gβγ are practically identical.

Biochemical characterization of purified GIRK channels have demonstrated that the GIRK4 and GIRK2 subunits can heteromultimerize with GIRK1 to form functional channels both in vitro and in vivo (Krapivinsky et al., 1995*a*; Liao et al., 1996; Corey et al., 1998). In functional studies using *Xenopus* oocytes, injection of GIRK1/GIRKx combinations of subunits yields significantly more current than any GIRK subunit injected alone or any other combination of GIRK2, 3, or 4 subunits (Duprat et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995*a*; Krapivinsky et al., 1995*b*; Dissmann et al., 1996; Velimirovic et al., 1996; Wischmeyer et al., 1997). A small amount of current is observed when GIRK1 is injected alone because it can heteromultimerize with the *Xenopus* GIRK homologue, XIR (Hedin, Lim & Clapham, 1996). In mammalian expression systems that do not in themselves possess endogenous GIRKs (e.g., CHO or COS7 cells), expressed GIRK1 did not localize to the cell membrane (Kennedy, Nemec, & Clapham, 1996) and was nonfunctional. In a heterologous expression system, all subunit combinations are possible. Since expression of GIRK1/GIRK4 in CHO cells reproduces the conductance, channel open time, and Gβγ sensitivity of native atrial I_{KACh} current, it is probable that the majority of channels formed have the native subunit stoichiometry.

The response to Gβγ was similar for GIRK1/GIRK3 channels and the native I_{KACh} channel studied previously. The Hill coefficients determined for the recombinant GIRK1/GIRK3 (1.5) and GIRK1/GIRK4 (1.5 channels in CHO cells were practically identical to the Hill coefficient determined for native GIRK1/GIRK4 (1.7) in rat atrial myocytes (Krapivinsky et al., 1995*c*). These Hill coefficients suggest that more than one Gβγ molecule binds per channel. Recent work examining changes in the distribution of channel open times of na-

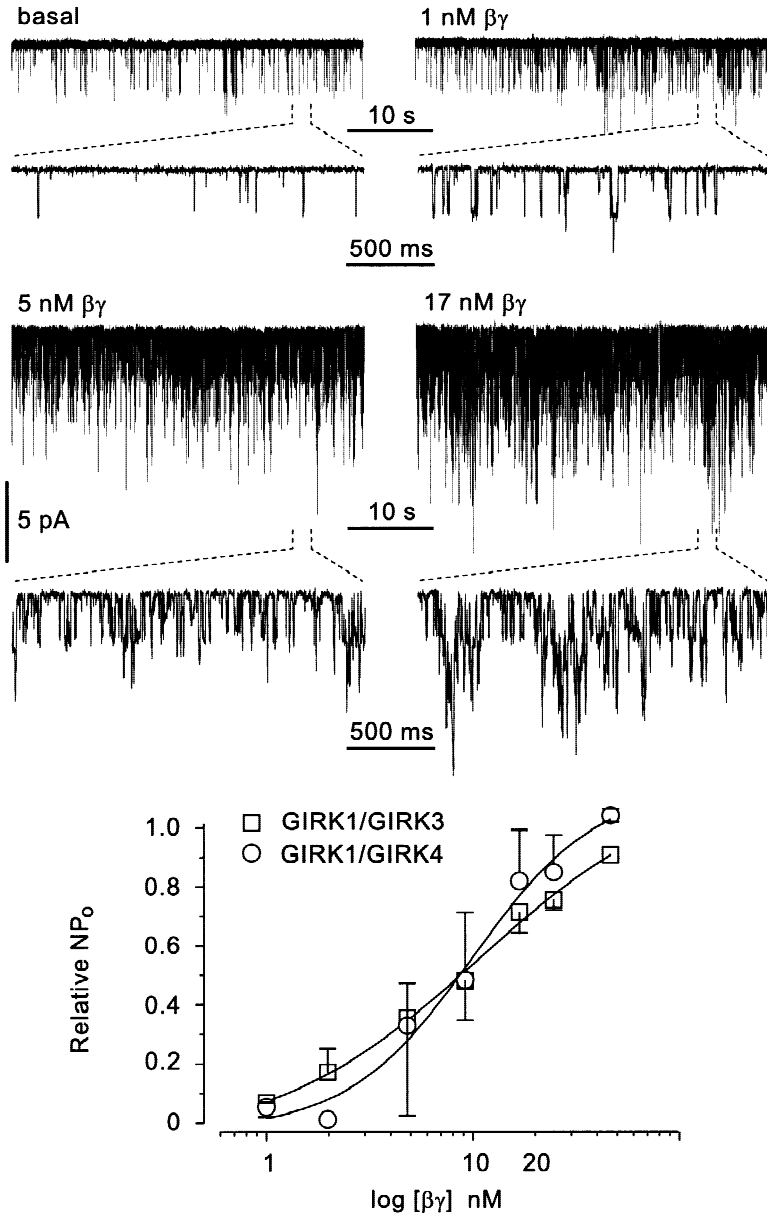

Fig. 4. GIRK1/GIRK3 channels and GIRK1/GIRK4 channels are activated by application of Gβγ to the cytoplasmic face of inside-out patches. The trace segments are from the recording of a single patch expressing GIRK1/GIRK3 channels and show the initial basal activity of the channel in the absence of Gβγ or GTPγS and the response of the patch to increasing concentrations of Gβγ. GIRK1/GIRK3 and GIRK1/GIRK4 channels activated in response to Gβγ with similar sensitivities as shown in the dose-response curves at bottom.

tive GIRK1/GIRK4 in atrial myocytes in response to Gβγ (Nemec, Wickman, & Clapham, 1999) also supports the hypothesis that more than one Gβγ molecule binds during channel activation.

In summary, GIRK1/GIRK2, 3, or 4 heteromultimers have indistinguishable single channel properties and Gβγ sensitivity. The pore region of the GIRKs is highly conserved (80–90% identity), but GIRK1 differs at four residues which are otherwise identical in GIRK2, 3, and 4. These four residues may play a role in determining the characteristic conductance of the GIRK1/GIRKx channels. One of these residues, phenylalanine 137 in GIRK1, has been shown to play an important role in determining the heteromultimeric channel conductance and opening kinetics (Chan et al., 1996; Wischmeyer et al., 1997). The *Xenopus* oocyte experiments also showed a general trend in the amount of current observed depending upon the GIRK subunit combination injected. The most current was observed with GIRK1/GIRK2, followed by GIRK1/GIRK4, and finally by GIRK1/GIRK3 (Duprat et al., 1995; Kofuji et al., 1995; Krapivinsky et al., 1995*a*; Krapivinsky et al., 1995*b*; Dissmann et al., 1996; Velimirovic et al., 1996; Wischmeyer et al., 1997). Since these channels have similar single channel conduc-

tances, differences in whole-cell current are probably due to differences in plasma membrane expression levels, which may be controlled by GIRK2, 3, and 4.

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