# A G-Protein-Activated Inwardly Rectifying $K^+$ Channel (GIRK4) from Human Hippocampus Associates with Other GIRK Channels

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Transcripts of a gene, *GIRK4*, that encodes for a 419-amino-acid protein and shows high structural similarity to other subfamily members of G-protein-activated inwardly rectifying $K^+$ channels (GIRK) have been identified in the human hippocampus. When expressed in *Xenopus* oocytes, GIRK4 yielded functional GIRK channels with activity that was enhanced by the stimulation of coexpressed serotonin 1A receptors. GIRK4 potentiated basal and agonist-induced currents mediated by other GIRK channels, possibly because of channel heteromerization. Despite the structural similarity to a putative rat $K_{ATP}$ channel, no ATP sensitivity or $K_{ATP}$-typical pharmacology was observed for GIRK4 alone or GIRK4 transfected in conjunction with other GIRK channels in COS-7 cells. In rat brain, GIRK4 is expressed together with three other subfamily members, GIRK1–3, most likely in identical hippocampal neurons. Thus, heteromerization or an unknown molecular interaction may cause the physiological diversity observed within this class of $K^+$ channels.

*Key words: inwardly rectifying $K^+$ channels; $K_{ATP}$ channels; GIRK; G-protein-activation hetero-oligomers; heterologous expression; hippocampus*

Inwardly rectifying $K^+$ ($K_{IR}$) channels allow a much larger $K^+$ influx than efflux and, thus, control the cell potential and diverse cellular functions without causing massive loss of $K^+$ ions (Hille, 1992; Jan and Jan, 1994). After the molecular cloning of the first $K_{IR}$ channel subtypes (Ho et al., 1993; Kubo et al., 1993a), it became obvious that this $K^-$ channel family was significantly different in structure from the classic voltage-dependent $K^+$ channels, which are composed of six membrane-spanning domains. $K_{IR}$ channels comprise only two transmembrane helices per subunit, which surround a putative pore region and N- and C-terminal cytoplasmic tails, suggesting an evolutionarily more primitive form of ion channel protein. The primary amino acid sequences of several channel species have been determined recently and may be classified as either steeply "voltage-dependent" IRK-type channels (Morishige et al., 1993; Bond et al., 1994; Koyama et al., 1994; Périer et al., 1994; Takahashi et al., 1994; Tang and Yang, 1994) or "mildly rectifying" and ATP-sensitive ROMK-type channels (Zhou et al., 1994; Takumi et al., 1995). In addition, three genes have been identified that likely encode $K_{IR}$ channels that are regulated by receptor-activated G-proteins (GIRK): expression cloning from rat heart atrium has led to the isolation of KGA/GIRK1 (Dascal et al., 1993a; Kubo et al., 1993b) and, more recently, two other structurally related channels, mbGIRK2 and mbGIRK3, were identified from the mouse brain (Lesage et al., 1994). *In situ* hybridization studies revealed that GIRK1 is expressed widely in rat brain (Karschin et al., 1994), as are mbGIRK2 and mbGIRK3 in mouse brain (Kobayashi et al., 1995), indicating an important role as a major target for G-protein-mediated receptor function and neuronal signal processing. This action likely is mediated by the stimulation of muscarinic $m_2$, $\alpha_2$ adrenergic, $D_2$ dopamine, histamine, serotonin 1A ($5\text{-}HT_{1A}$), $A_1$ adenosine, $GABA_B$, $\mu$-, $\kappa$- and $\delta$-opioid, somatostatin, and possibly other $G_{i/o}$ protein-coupled receptors (North, 1989; Hille, 1992). Interestingly, single-channel analysis revealed some physiological inhomogeneities among the channel proteins that have been proposed to be G-protein-activated (Pennefather et al., 1987; Yatani et al., 1987; vanDongen et al., 1988; Pennington et al., 1993). At present, it is still uncertain whether these differences are attributable to the differential distribution of GIRK subfamily members, on splice variants that may exist in the brain, on the formation of hetero-oligomeric polypeptides, or on cell-specific regulation by accessory proteins.

In this report, we demonstrate the molecular cloning and cellular localization of a fourth type of GIRK channel that coexists and may associate with hetero-oligomeric channels together with GIRK1–3 transcripts in the human hippocampus.

## MATERIALS AND METHODS

*Molecular cloning of* GIRK4. For genomic analysis, homology screening with a rat GIRK1 probe (Dascal et al., 1993b) was performed on a human leukocyte genomic DNA library (Clontech, Palo Alto, CA) under low-stringency conditions according to conventional protocols (Sambrook et al., 1990). A total of $10^6$ clones was hybridized overnight with the [$^{32}$P]*d*-cytidine triphosphate randomly labeled 2.1 kb GIRK1 cDNA fragment at 42°C in a buffer containing 30% formamide, 5× SSC, 5× Denhardt's reagent, 0.1% SDS, 20 mM $NaPO_4$, and 100 $\mu$g/ml herring sperm DNA. Filters were rinsed twice in 2× SSC/0.1% SDS and washed once at 37 and 65°C for 15 min each with 0.1× SSC/0.5% SDS. Nineteen positive clones between 12 and 20 kb in length were isolated and analyzed by restriction mapping and Southern blotting. Sequence analysis of the two fragments showing the strongest autoradiography signals revealed complete open reading frames (ORFs) of a GIRK-like gene, *hg*GIRK4, including an intronic sequence of ~5.5 kb starting at base 937 and 5′- and 3′-untranslated regions (UTRs) of >500 bp in length. For comparison, an

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~360 bp fragment spanning the single 5′-exon–intron splice junction in the corresponding human sequence was PCR-amplified from rat genomic DNA using primers derived from *hg*GIRK4.

A 321 bp fragment encoding the C-terminal 107 amino acids of the *hg*GIRK4 ORF then was used as a probe to screen a human hippocampus cDNA library (Stratagene, La Jolla, CA) under the above conditions. Of $5 \times 10^5$ filter-screened clones, 13 positive clones were excised from the λZAP II vector into pBluescript SKII (both from Stratagene) and analyzed by restriction digestion and Southern blotting. Sequencing of the cDNA clone showing the strongest signal identified a clone, incomplete in the 5′-ORF but 100% identical to *hg*GIRK4 in the C-terminal 239 amino acids. For expression, a clone termed GIRK4 was assembled in pSP72 (Promega, Madison, WI) that contained 90 bp of 5′-UTR plus 915 bp of ORF from *hg*GIRK4 and a human hippocampal cDNA fragment delivering the C-terminal 345 bp of the ORF plus 842 bp of 3′-UTR.

Human GIRK1, GIRK2, and GIRK3 channel orthologs were isolated from the same human hippocampus cDNA by homology screening with a rat GIRK1 probe under low-stringency conditions as described above. All identified clones were sequenced completely on both strands using the prism Sequenase dye-terminator kit on an automatic sequencer (Applied Biosystems, Foster City, CA). Sequence analysis and alignments were performed using the LASERGENE software (DNAstar, Madison, WI) on a Macintosh computer; National Center for Biotechnology Information nucleotide database searches were performed with software from the Genetics Computer Group (Madison, WI).

*Expression and electrophysiological analysis of* GIRK4. Two micrograms of the transcription vector pSGEM (a gift from Dr. M. Hollmann, Göttingen, Germany) containing GIRK4, GIRK1, or the human 5-$HT_{1A}$ receptor (Kobilka et al., 1987) were linearized and transcribed *in vitro* using the TransProbe T Kit (Pharmacia, Piscataway, NJ). *Xenopus laevis* oocytes were defolliculated by collagenase treatment as described previously (Methfessel et al., 1986) and injected with ~6 ng of GIRK channel cRNA and, where applicable, ~3 ng of 5-$HT_{1A}$ receptor cRNA. Oocytes were incubated for 2–7 d at 19°C in ND96 solution [(in mM) NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, pH 7.4–7.5] supplemented with 100 μg/ml gentamicin and 2.5 mM sodium pyruvate.

Two-electrode voltage-clamp measurements were performed with a TURBO TEC-10 C amplifier (NPI, Tamm, Germany) and sampled through an EPC9 (Heka Electronics, Lamprecht, Germany) interface using PULSE/PULSEFIT software (Heka). For rapid exchange of external solutions, oocytes were placed in a small-volume perfusion chamber with a constant flow of ND96 or "high $K^+$" solution [(in mM) KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5, pH 7.4–7.5] supplemented with the respective agonist or drug as indicated.

cDNA clones also were subcloned in the eukaryotic expression vector pSVSport 1 (Life Technologies, Gaithersburg, MD). Confluent simian SV-40-transformed COS-7 kidney cells (ATCC CRL1650), grown on glass coverslips, were transfected with 0.8 μg/ml (GIRKs) and 0.4 μg/ml medium (5-$HT_{1A}$ receptor) of the expression plasmids using LipofectAmine and Opti-MEM I (Life Technologies) according to the manufacturer's protocol. Whole-cell and single-channel recordings in the cell-attached and excised-patch configuration (Hamill et al., 1981) were performed at room temperature 48–72 hr after transfection in a bath solution consisting of (in mM): NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 5, pH 7.4. Patch pipettes were pulled from borosilicate glass capillaries (Kimble Products, Sussex, UK), SYLGARD-coated (Dow Corning, Corning, NY), and heat-polished to give input resistances of 3–7 MΩ (whole-cell) and 7–9 MΩ (single-channel). The intracellular recording solution contained (in mM): KCl 140, $MgCl_2$ 2, EGTA 1, $Na_2$-ATP 1, cAMP 100, GTP 100, and HEPES 5, pH 7.3. All chemicals were provided by Sigma (Deisenhofen, Germany) unless indicated otherwise. A motor-driven fast-microperfusion system was used to perform local solution changes (in <30 msec) near the measured cell. Currents were recorded with an EPC9 (Heka) patch-clamp amplifier and low-pass-filtered at 1–2 kHz. Stimulation and data acquisition were controlled by the PULSE/PULSEFIT software package (Heka) on a Macintosh computer, and data analysis was performed with IGOR software (WaveMetrics, Lake Oswego, NY). Data are presented as mean ± SD (number of cells).

In situ *hybridization.* Adult Wistar rats were anesthetized and decapitated, and the tissue was removed and quickly frozen on dry ice. Brain and heart sections were cut on a cryostat at 12–16 μm, thaw-mounted onto Superfrost Plus slides, fixed with 4% *p*-formaldehyde in PBS, pH 7.4, dehydrated, and stored under ethanol until hybridization. Antisense and sense oligonucleotides of 45–50 bases in length were chosen from rat GIRK4 N-terminal (amino acids 3–16 and 32–46) and C-terminal (amino acids 375–391) regions with the least homology to other known $K_{IR}$ channels. Oligonucleotides were 3′-end-labeled with [$^{35}$S]dATP (DuPont NEN, 1200 Ci/mmol) by terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) and used for hybridization at concentrations of 2–10 pg/μl (400,000 cpm/100 μl hybridization buffer/slide). Alternatively, antisense and sense cRNA probes were generated with T3 and T7 RNA polymerase, respectively, by *in vitro* transcription using [$^{35}$S]uridine triphosphate (DuPont), from an ~580 bp fragment covering the C-terminal region of the rat GIRK4 ORF.

Overnight hybridizations with sense and antisense cRNA probes (0.02–0.2 ng/μl) were carried out as described previously (Karschin et al., 1994). For hybridizations with labeled oligonucleotides, slides were air-dried and hybridized overnight at 43°C in 100 μl of buffer containing 50% formamide, 10% dextran sulfate, 50 mM dithiothreitol, 0.3 M NaCl, 30 mM Tris-HCl, 4 mM EDTA, 1× Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.5 mg/ml polyadenylic acid. After hybridization, slides were washed at high stringency, dehydrated, and exposed to

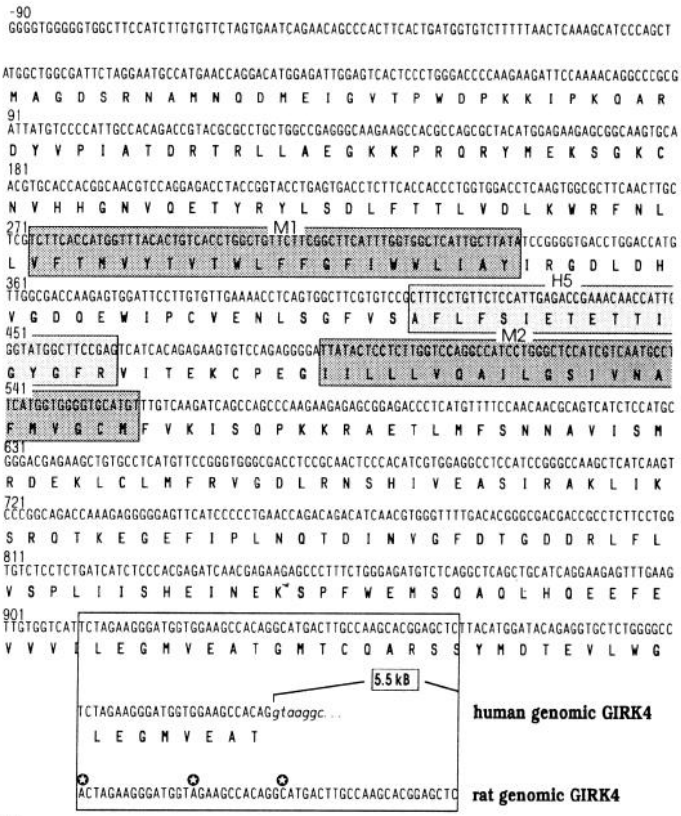


*Figure 1.* Nucleotide and deduced amino acid sequence of the human GIRK4. The sequence of the genomic GIRK4 is identical in the ORF to the hippocampal cDNA clone and is shown with 90 bases of 5′-UTR. The partial alignment to the homologous rat genomic sequence in the C-terminal ORF region shows a 5′-splice junction at 937 bp for the human gene followed by an intronic sequence of ~5.5 kb in length. *Stars in circles* in this region indicate base-pair exchanges in the corresponding rat gene. The ORF codes for a protein of 419 amino acids with two predicted transmembrane regions, M1 and M2, and a putative pore-forming structure, H5 (*boxed segments*). S57, T158, and S233 are consensus sites for protein kinase C phosphorylation; a single N-linked glycosylation site is located at N132. Amino acids are indicated in the single-letter code. The Genbank accession number for the sequence is L47208.

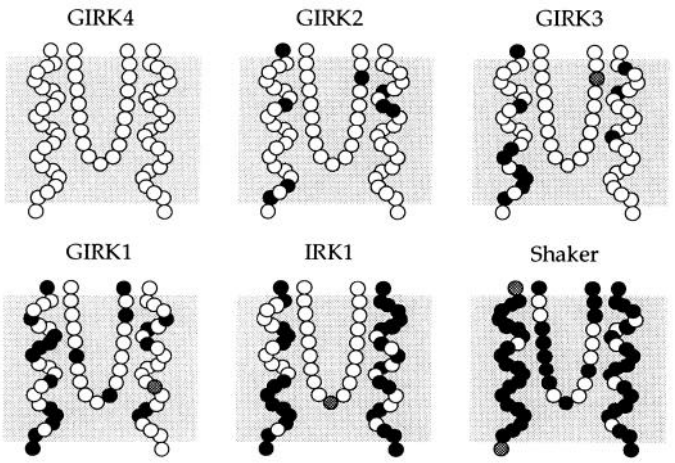


*Figure 2.* Structural models of the proposed membrane-spanning and pore-forming domains of the human GIRK4, GIRK1–3, rat IRK1 (Wischmeyer et al., 1995), and the *Drosophila* B channel of *Shaker* (Tempel et al., 1987). *Open circles* represent amino acids identical with, and *solid circles* show residues different (*gray symbols* represent non-conserved exchanges) from GIRK4. The amino acid sequence identities to GIRK4 in the region shown are 89% (*GIRK2*), 83% (*GIRK3*), 68% (*GIRK1*), 53% (*IRK1*), and 21% (*Shaker*). For *GIRK4*, the following sites have been chosen as domain boundaries: M1 (V92/Y113); H5 (A139/R155); and M2 (I165/M186).

Kodak Biomax x-ray film (Rochester, NY) for 4–14 d. For cellular resolution, selected slides subsequently were dipped in photographic emulsion (NTB 2, Kodak), exposed for 4–12 weeks, and developed in Kodak D-19 for 3 min.

## RESULTS

### Molecular biology

Homology screening of a human leukocyte genomic DNA library with a radiolabeled GIRK1 cDNA fragment revealed a genomic clone with an ORF that coded for a 419-amino-acid polypeptide with the typical structural motifs of other known $K^+$ inward rectifiers (Fig. 1). A conserved putative pore-forming region, H5, is flanked by two transmembrane-spanning segments, M1 and M2; an N-linked glycosylation site at $Asn^{132}$ between M1 and H5 suggests that the N and C termini of the protein are located at the intracellular side of the membrane. The human gene that we termed *GIRK4* was similar (93% amino acid identity) to a "rat cardiac $K_{ATP}$" ($rcK_{ATP}$) channel that has been isolated recently from rat cardiac tissue and found to express ATP-sensitive $K^+$ channels in human kidney HEK293 epithelial cells (Ashford et al., 1994). In contrast to the equivalent rat gene, which we PCR-amplified from rat genomic DNA, the human gene (*hg*GIRK4) was interrupted by a single intronic sequence at $Thr^{312}$ in the middle of the C-terminal tail region. Figure 1 shows that, with respect to the rat sequence, the exchange of a single base at location 938 in the human gene reveals a classic 5′-exon–intron splice consensus sequence "AG/GTAAG." The intron was partially sequenced and found to be ~5.5 kb in size.

Using a C-terminal fragment of *hg*GIRK4 for homology screening, GIRK4 also was identified from a human hippocampal cDNA library. In addition, in a parallel screening series of the same library with a partial rat GIRK1 probe, three different but closely related types of cDNA clones were identified. They likely were the human orthologs of the rat heart GIRK1 (97% amino acid identity; Kubo et al., 1993b), mouse brain mbGIRK2 (97% amino acid identity; Lesage et al., 1994), and mbGIRK3 (97% amino acid identity; Lesage et al., 1994), which suggests that mRNA transcripts of four GIRK subfamily members were present in human hippocampal tissue. The overall sequence identity between the human GIRK4 and GIRK1 was 53%; it was 66% for GIRK3 and 69% for GIRK2. Smaller sequence homologies were found between *GIRK4* and members of the IRK (43% to rat IRK1) and ROMK-family (38% to rat ROMK1). The striking structural similarities between the different putative members of the subfamily of GIRK channels were even more pronounced in a comparison of the conserved membrane/pore domains as shown in Figure 2. The amino acid sequences in this region were dissimilar to IRK-type $K^+$ inward rectifiers and highly different from classic voltage-dependent $K^+$ channels. Of particular interest was the >85% amino acid identity between *hg*GIRK4 and the human GIRK2 counterpart in most of the C-terminal region that is likely to be involved in G-protein binding and blocking of the channel pore (Takao et al., 1994; Pessia et al., 1995).

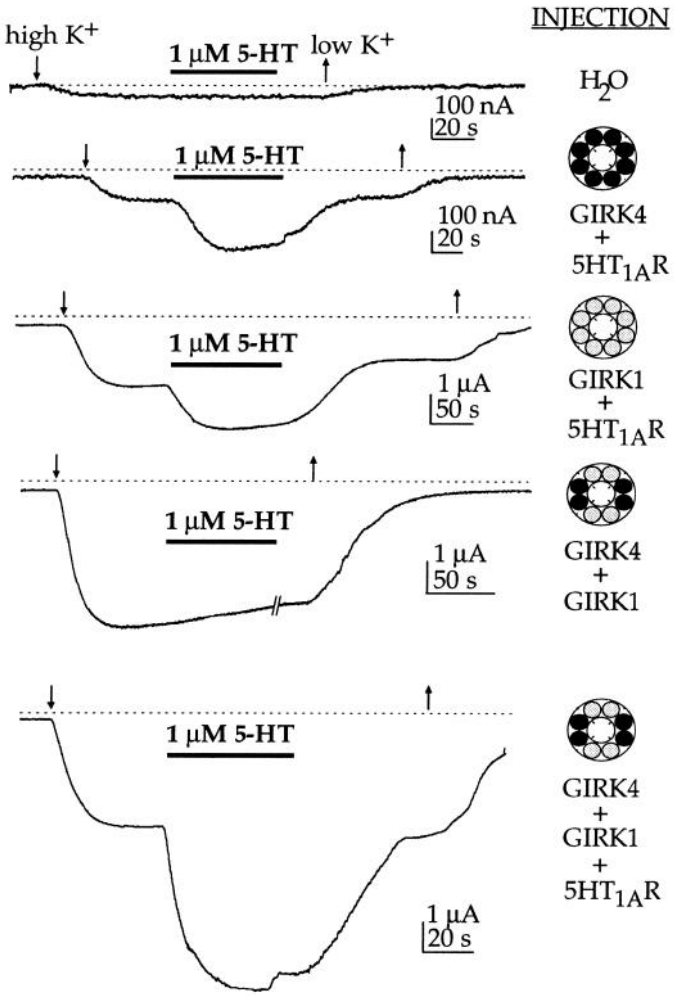


*Figure 3.* Expression of GIRK4 and GIRK1 channels elicits basal and agonist-dependent $K^+$ current in *Xenopus* oocytes. Oocytes were injected with cRNA of GIRK4, GIRK1, or a combination of both in the absence or presence of the human $5\text{-}HT_{1A}$ receptor as indicated on the *right*. High $K^+$ (96 mM), low $K^+$ (2 mM) solution, and 1 μM 5-HT were applied to the bath (*arrows* and *solid bars*) to the oocytes voltage-clamped at −80 mV. *Dashed lines* indicate the zero-current levels. Models of the channel pores are for illustration only.

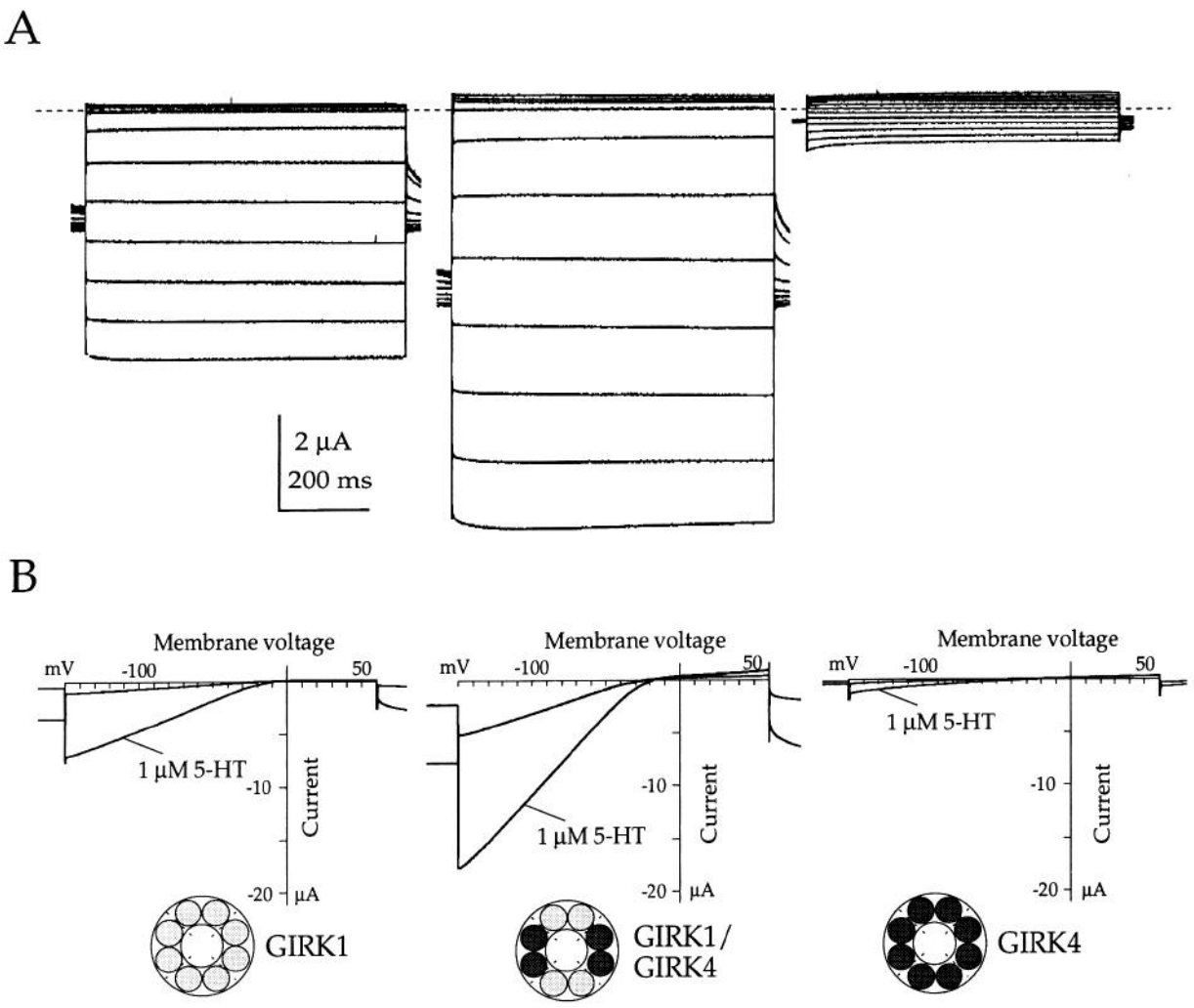


*Figure 4.* Coexpression in *Xenopus* oocytes of GIRK4/GIRK1 channels potentiates currents mediated by either GIRK4 or GIRK1 alone. Oocytes were injected with identical (0.6 ng each) amounts of cRNA for GIRK1, GIRK4, or GIRK1/GIRK4. Currents are 5-HT-induced responses of oocytes clamped to step potentials between −150 and +70 mV in 20 mV increments from a holding potential of −80 mV (*top traces*), and to voltage ramps of 500 msec duration between −150 and +60 mV (*bottom traces*). Response amplitudes are from oocytes of one preparation and are representative of many cells (see text). Models of homo- and heteromeric channels are for illustration only.

### Functional expression of GIRK4

Mouse brain mbGIRK2 had been classified as a GIRK-type channel because it was activated by coexpressed G-protein-coupled δ-opioid receptors in *X. laevis* oocytes (Lesage et al., 1994). To demonstrate that GIRK4 also functionally belongs into this subfamily, we characterized the properties of GIRK4 expressed in *Xenopus* oocytes. Injection of GIRK4 cRNA induced the expression of a basal (agonist-independent, measured in 96 mM high-$K^+$) inwardly rectifying $K^+$ current that was 275 ± 124% ($n$ = 7) larger in amplitude compared with currents measured in $H_2O$-injected oocytes ($n$ = 8; Fig. 3). When human G-protein-coupled 5-$HT_{1A}$ receptors (5-$HT_{1A}$R) were coexpressed with GIRK4, bath application of 1 μM 5-HT yielded an additional inwardly rectifying $K^+$ current component. With oocytes voltage-clamped at −80 mV in high-$K^+$ solution, peak current amplitudes were −182 ± 82 nA in the absence and −482 ± 122 nA in the presence of 5-HT ($n$ = 7).

Because heteromeric polypeptide formation was suggested to occur in the heart for muscarinic $K_{ACh}$ channels (Krapivinsky et al., 1995), we investigated the coexpression of the human GIRK4 with rat brain GIRK1 channel subunits in oocytes. To allow for a direct comparison of basal and 5-HT-dependent peak currents, oocytes were injected with equal amounts of cRNA encoding GIRK4, GIRK1, or a combination of both. Coexpression of cRNA encoding GIRK1, GIRK4, and the 5-$HT_{1A}$R caused large, sometimes slowly desensitizing (cf. Kovoor et al., 1995) inwardly rectifying $K^+$ currents (−4.92 ± 2.03 μA, in high-$K^+$ solution; $n$ = 9) that were severalfold larger than for GIRK4 (see above) or GIRK1 (−1.03 ± 0.79 μA, $n$ = 7) alone (Fig. 3). This current was enhanced dramatically by perfusion with 1 μM 5-HT ($n$ = 7) elicited by GIRK1 alone and returned to basal levels after removal of the agonist. Thus, GIRK1/GIRK4 coexpression caused an ~3- and 23-fold current increase compared with GIRK1 and GIRK4 alone, respectively (Fig. 4). However, the overall amplitude relationship between the basal current and the 5-HT-induced current varied between 15 and 60%, independent of the total current amplitude or time period between injection and data acquisition.

Both the 5-HT-induced and the basal current component were dependent on the extracellular $K^+$ concentration ($[K^+]_o$) and

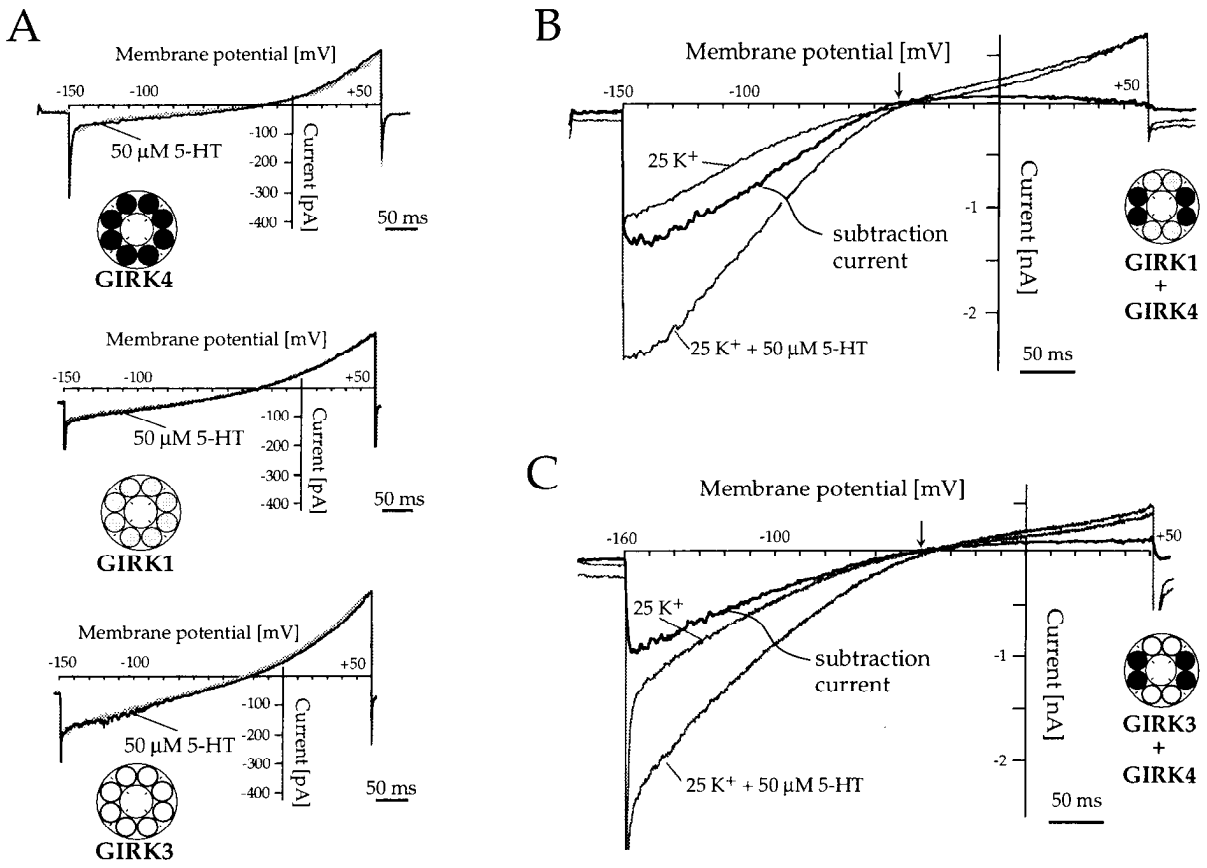


*Figure 5.* Only coexpression with GIRK4 subunits causes inward $K^+$ currents in COS-7 cells. All COS-7 cells were transfected with 5-$HT_{1A}$ and cotransfected with GIRK4, GIRK1, or GIRK3 alone (*A*), with GIRK1 and GIRK4 (*B*), and with GIRK3 and GIRK4 (*C*). Currents are whole-cell responses to voltage ramps of 500 msec duration between −160 and +60 mV. Solutions containing 25 mM $K^+$ with or without 50 μM 5-HT are applied with a fast-microperfusion system as indicated by the *arrows* and *solid bars*. GIRK4, GIRK1, or GIRK3 (*A*) by themselves do not reveal detectable current responses; however, in GIRK1/GIRK4 (*B*)- and GIRK3/GIRK4 (*C*)-transfected cells, application of 25 mM $K^+$ elicits a basal inward current ($V_H$ = −120 mV) that is increased by application of 50 μM 5-HT. Subtraction of ramp currents recorded in the presence or absence of 50 μM 5-HT reveals an inwardly rectifying current that reverses close to the Nernst potential for $K^+$.

were sensitive to block by 100–300 μM $BaCl_2$ (data not shown). Oocytes injected with $H_2O$ (see above) or the 5-$HT_{1A}$R ($n$ = 5) alone did not reveal any significant current responses—nor did oocytes that were injected only with GIRK1 and GIRK4 cRNAs ($n$ = 6) show a 5-HT-induced component in addition to the basal, inwardly rectifying current ($I_{hK}$) observed in high-$K^+$ solution (Fig. 3).

In contrast to *Xenopus* oocytes, GIRK4 transfected together with 5-$HT_{1A}$R in HEK293 cells (data not shown) and COS-7 cells ($n$ = 27) did not reveal any macroscopic currents different from untransfected or mock-transfected cells ($n$ = 7 each). Also, we and others (Kofuji et al., 1995; Krapivinsky et al., 1995; Philipson et al., 1995) failed in attempts to achieve functional expression of the rat GIRK1 cDNA in COS-7 ($n$ = 34) and other mammalian host cells (Chinese hamster ovary, HEK293). However, when GIRK1, GIRK4, and 5-$HT_{1A}$R were expressed in COS-7 cells via an SV-40-based vector in triple transfections, all cells ($n$ = 42) that were determined to be transfected from visual inspection exhibited a basal, agonist-independent, inwardly rectifying $K^+$ conductance ~13-fold larger than untransfected cells (Fig. 5*A,B*). Local perfusion with a saturating 5-HT concentration (50 μM) increased the inward current in 32 of 42 cells by 86 ± 29%. As found in oocytes, a change in $[K^+]_o$ caused the reversal potential of the 5-HT-induced current to follow the Nernst potential ($E_K$) for $K^+$, suggesting $K^+$ selectivity of the channel pores (Fig. 5*B,C*). Moreover, $Ba^{2+}$ induced a concentration- and voltage-dependent current block in all cells tested ($n$ = 10). Characteristically, the time constants for the onset of the $Ba^{2+}$ inhibition (1 mM) were markedly faster ($\tau_{on}$ = 197 ± 125 msec) than those for relief of inhibition ($\tau_{off}$ = 7.67 ± 2.65 sec), indicating slow $Ba^{2+}$ unbinding from the pore, as observed for other $K^+$ inward rectifiers (Wischmeyer et al., 1995).

Preliminary attempts to functionally express GIRK3 cDNA isolated from rat brain (E. Dißmann, unpublished data) revealed results qualitatively similar to those found for the coexpression of GIRK1/GIRK4. Rat brain GIRK3 did not show functional expression in COS-7 cells when transfected alone (coexpressed with 5-$HT_{1A}$R), but gave rise to significant basal and 5-HT-elicited inwardly rectifying $K^+$ currents when cotransfected together with GIRK4 and 5-$HT_{1A}$R (Fig. 5*C*).

Because GIRK4 was found to be structurally similar to what has been referred to an $rcK_{ATP}$ (Ashford et al., 1994), we investigated whether GIRK1/GIRK4 could exhibit pharmacological properties characteristic of native $K_{ATP}$ channels. In whole-cell measurements of GIRK1/GIRK4-transfected COS-7 cells, the sulfonylurea $K_{ATP}$ channel blocker glibenclamide (Edwards and Weston, 1993) affected neither the basal nor the 5-HT-induced channel conductance when applied at a concentration of 50 μM ($n$ = 4). Similarly, application of 50 μM pinacidil, a $K_{ATP}$ channel opener (Quast and Cook, 1989), did not increase the macroscopic cur-

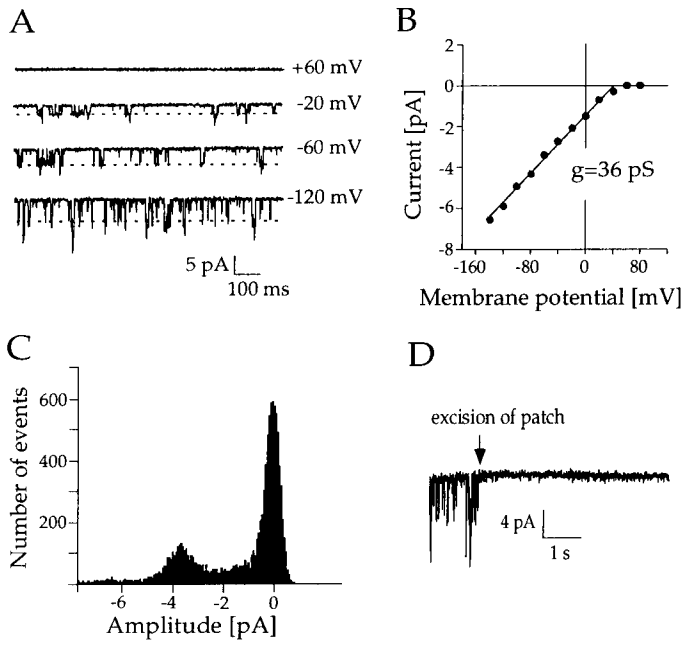


*Figure 6.* Cell-attached single-channel recordings of GIRK4/GIRK1 channels expressed in COS-7 cells. *A*, Traces with elementary currents are shown from one patch at the selected voltages indicated on the *right* with 140 mM $K^+$ in the pipette. The potentials shown represent the pipette potentials that are shifted to more negative potentials by the resting potential of the cell. *B*, Current–voltage plot (pipette potential on the *abscissa*) of the single-channel currents in *A* reveals a slope conductance ($g_{GIRK4/GIRK1}$) of 36 pS; the *straight line* was fitted by linear regression. *C*, Amplitude histogram with the number of events plotted against current amplitude (calculated for single-channel events recorded at $V_H = -80$ mV under the same conditions as above). *D*, Patch excision into the inside-out configuration causes GIRK4/GIRK1 channel activity to disappear instantaneously irrespective of the presence of ATP (1 mM) in the bath solution. All current records were filtered and digitized at 1 kHz.

rents ($n = 7$). Native $K_{ATP}$ channels open when intracellular ATP concentrations are decreased (Noma, 1983; Cook and Hales, 1984); however, after omission of ATP from the pipette solution, no increase in basal or 5-HT-induced current over time ($n = 3$) was observed.

In the cell-attached recording configuration, single-channel activity of $K_{IR}$ channels with a unitary slope conductance of ~36.3 ± 0.7 pS ($n = 6$) was detected in GIRK1/GIRK4-cotransfected cells, but this activity was never recorded in either untransfected/mock-transfected COS-7 cells or cells transfected with either of the two GIRK subunits alone ($n > 23$; Fig. 6). Channels appeared to be similar in unitary conductance and open probability to recombinant "cardiac inward rectifier" (CIR)/GIRK1 (Krapivinsky et al., 1995) and $K_{ACh}$ channels in rat atrial myocytes. Single-channel activity disappeared instantaneously after patch excision into the inside-out configuration irrespective of the ATP concentration (0–1 mM) in the bath solution (Fig. 6*C*). Local perfusion with 100 μM GTPγS in the presence 1 mM ATP after current run-down was found to restore channel activity.

### Cellular localization of GIRK4

Because human central nervous system (CNS) tissue appropriate for *in situ* hybridizations was difficult to obtain, we examined the distribution of GIRK4 in tissue sections from the rat with both radiolabeled cRNA and synthetic oligonucleotides of the rat GIRK4 homolog. In heart, high mRNA expression levels were found only in the atrium, but not in the ventricle (Fig. 7*F*). In contrast, the general expression level of GIRK4 was very low in most regions of the CNS with both types of probes when compared with the abundant expression of GIRK1 (Karschin et al., 1994) and GIRK2/GIRK3 (C. Karschin, unpublished data) in the rat brain. Overall, mainly the hippocampus and superior colliculus appeared positive on x-ray films, but examination of emulsion-dipped slides also revealed some distinct hybridization signals in other brain areas. In the hippocampal formation, expression was prominent in CA3 pyramidal neurons but only moderate to low in both CA1 neurons and dentate gyrus granule cells (Fig. 7*A,B*). Many cells in the subiculum and entorhinal cortex of the hippocampal formation also were found to be positive. In the major target of retinal ganglion cells, the superior colliculus, the optic nerve layer–intermediate gray layer contained various strongly labeled cells (Fig. 7*D*). In the medial habenular nucleus, a majority of cells were found to be positive, whereas only a few neurons were strongly labeled in both the medial vestibular nucleus (Fig. 7*E*) and the septal nucleus. Diffuse hybridization signal also was observed in the inferior olive, the paraventricular thalamic, and the ventromedial–hypothalamic nucleus. In the cerebellar cortex, only the large Purkinje cells revealed positive hybridization signals (Fig. 7*B*).

## DISCUSSION

Based on the >93% amino acid sequence identity, the novel G-protein-activated $K^+$ inward rectifier GIRK4 (or Kir3.4; for the alternative terminology, see Doupnik et al., 1995) likely represents a human ortholog of the $rcK_{ATP}$ (Ashford et al., 1994) and CIR (Krapivinsky et al., 1995), both isolated from rat heart. An uncharacterized clone of identical sequence from a human pancreatic library has been deposited in Genbank (accession number X83582). By isolating GIRK4 from a human hippocampal cDNA library, we showed that GIRK4 transcripts not only were not confined to cardiac tissue but were present in the CNS, as suggested for $rcK_{ATP}$ from reverse transcription-PCR (Ashford et al., 1994). *In situ* hybridizations on rat tissue performed in this report demonstrated only low to moderate RNA expression levels of GIRK4 in the CNS, with few areas (e.g., every single pyramidal neuron in the hippocampal CA3 region) of strongly labeled cells. In contrast, abundant expression has been found for each of the other subfamily members GIRK1–3 throughout the rat brain (Karschin et al., 1994) (C. Karschin, unpublished data).

When expressed in *Xenopus* oocytes and COS-7 cells together with $5\text{-}HT_{1A}R$, GIRK4 functionally assembled to $K_{IR}$ channels that were subject to activation by 5-HT. In the mammalian brain, $5\text{-}HT_{1A}R$ elicit IPSPs, possibly via GIRK channels in CA1 pyramidal neurons of the rat hippocampus (Andrade et al., 1986). The functional reconstitution of GIRK channel activation by coexpressed G-protein-coupled receptors indicated that, in both recombinant cell systems, endogenous G-proteins adequately completed the signaling cascade. The host cells also may have contributed to the expression of the agonist-independent "basal $K^+$ current" component that became particularly pronounced in elevated $[K^+]_o$. Dascal et al. (1993a) argued that this "$I_{hK}$" current, because of its lower $Ba^{2+}$ sensitivity, was different from the basal activity of the same GIRK channels that were activated by the agonist. Although we found significant variations in the amplitude ratios of $I_{hK}$ and the 5-HT-induced current, both components were potentiated equally by coexpression of GIRK1/GIRK4. This suggests that $I_{hK}$ indeed was dominated by a GIRK current, the stimulation of which likely was attributable to a basal

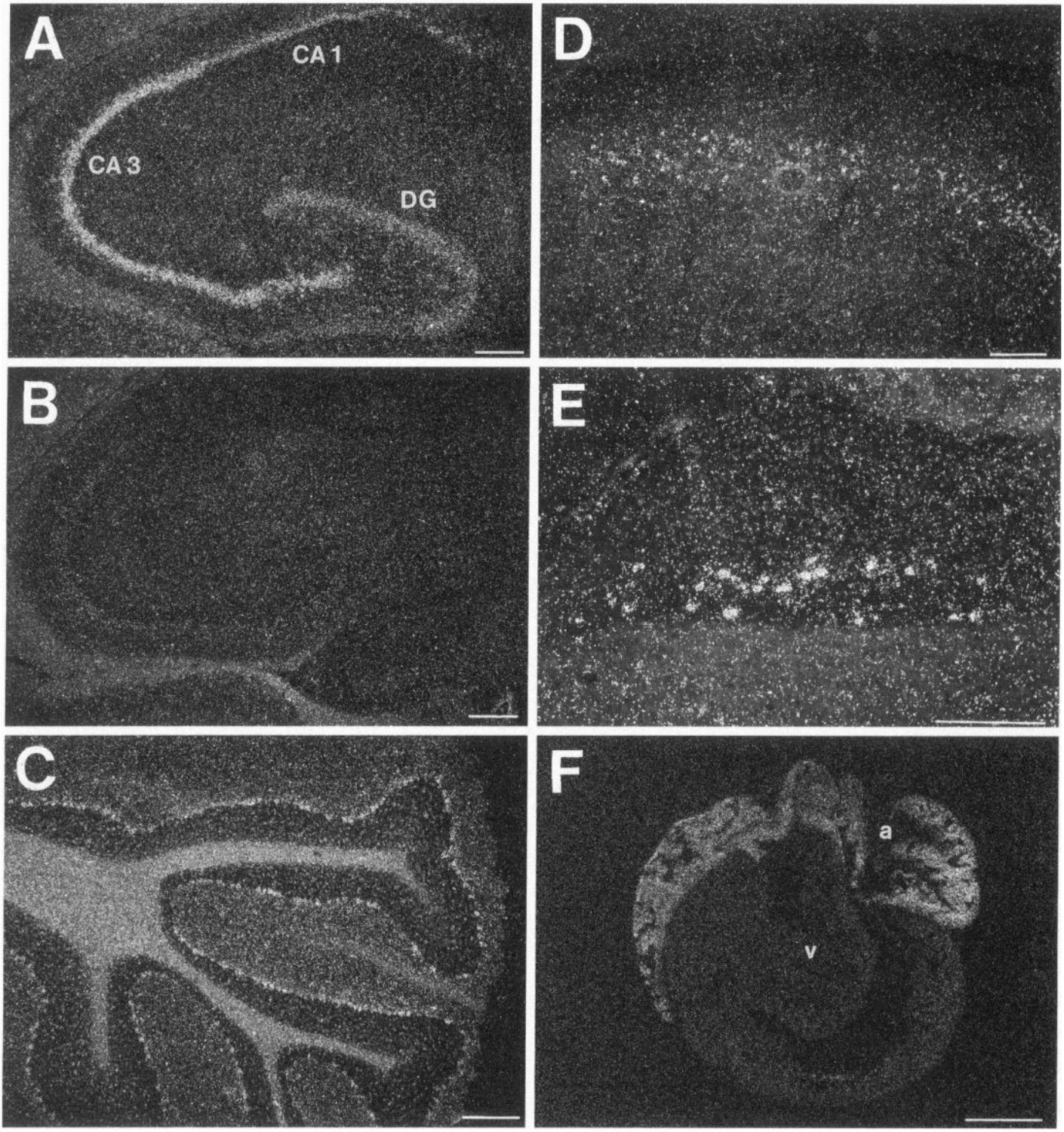


*Figure 7.* Localization of GIRK4 in rat tissue as revealed by *in situ* hybridization. Sections were hybridized with cRNA probes (*A–C*) or oligonucleotide probes (*D–F*) of GIRK4 as described in Materials and Methods. *A*, Dark-field photomicrograph of a horizontal section through the hippocampal region shows strong labeling in CA3 pyramidal neurons of Ammon's horn (*CA 3*), and moderate to low expression levels in the CA1 and dentate gyrus (*CA 1, DG*) regions. *B*, Sense RNA control in an adjacent section. *C*, Purkinje cells in the cerebellar cortex are labeled strongly; oligodendroglia in the cerebellar white matter do not accumulate silver grains. A few distinct neurons in the optic nerve layer–intermediate white layer of the superior colliculus (*D*) and in the medial vestibular nucleus (*E*) are also positive. Emulsion-dipped sections were exposed for 80 d. *F*, X-ray-film image of a horizontal rat heart section showing strong expression of GIRK4 mRNA predominantly in the atrium (*a*) but not in the ventricle (*v*). Exposure time, 8 d. Scale bars: *A–E*, 250 μm; *F*, 2 mm.

level of activated G-protein subunits (see also Kovoor et al., 1995).

As has been demonstrated for classic voltage-dependent A-type $K^+$ channels in the brain (Sheng et al., 1993; Wang et al., 1993), coassembly between homologous subunits into heteromultimers also may occur in the family of $K_{IR}$ channels. Recently, it was demonstrated that the native G-protein-activated $K^+$ channel $K_{ACh}$, well known as the target for the muscarinic action in the mammalian heart, was comprised of the two homologous subunits, GIRK1 and the rat CIR (Krapivinsky et al., 1995). Comparable with the results obtained for the rat CIR/GIRK1 coexpression, we found that the human GIRK4 also "associated" with other members of the GIRK family (GIRK1, GIRK3) to yield whole-cell $K^+$ currents that were potentiated compared with those of the putative homomeric channels alone. Interestingly, coexpression of GIRK4 and GIRK1 in mammalian COS cells revealed prominent whole-cell currents, whereas we were unable to record 5-HT-induced currents in cells transfected with either of them individually. This apparent discrepancy between oocytes and a mammalian host cell may indicate that the cell-specific environment somehow influences protein assembly/channel activity of GIRK channels. Therefore, whenever GIRK-type channel subunits yield $K^+$ channels, caution must be exerted when determining whether functional polypeptides are caused by a molecular association of the transfected/injected subunits with channel subunits that occur endogenously in different cell types.

It remains to be determined whether any of the newly isolated channels represent the long-sought $K_{ATP}$ channel. Ashford et al. (1994) reported that $rcK_{ATP}$ (hence the name), expressed in the human HEK293 and hamster BHK21 kidney epithelial cell lines, produced classic $K_{ATP}$ channels of ~70 pS unitary conductance that were activated by the $K^+$ channel opener pinacidil and that the open probability of which decreased in the presence of micromolar concentrations of intracellular ATP. In the cDNA, no obvious structural motifs of a Walker A-type ATP-binding site, as seen in ROMK channels, could be identified. When expressed in *Xenopus* oocytes, neither the identical CIR nor the human ortholog GIRK4 showed any sensitivity to millimolar ATP concentrations, regardless of whether it was expressed alone or in conjunction with GIRK1. In addition, CIR/GIRK4 channels alone (15–30 pS) or together with GIRK1 (~36 pS) revealed polypeptides with a unitary conductance significantly smaller than the 70 pS conductance typical of most $K_{ATP}$ channels in native tissues (Ashcroft and Ashcroft, 1990). Furthermore, preliminary efforts failed to reconstitute the molecular association between the putative $K_{ATP}$ channel subunit $rcK_{ATP}$/CIR and the recently cloned sulfonylurea receptor, SUR (Aguilar-Bryan et al., 1995). From these data, and the distinct mRNA expression in heart and brain shown here, we conclude that the protein that confers ATP sensitivity to native $K_{ATP}$ channels is unlikely to be CIR/GIRK4. Still another potential $K_{ATP}$ candidate is the recently identified $uK_{ATP}$-1 channel that is ubiquitously expressed in rat pancreas, skeletal muscle, heart, and pituitary gland and shows only 44% amino acid identity to $rcK_{ATP}$/CIR (43% to GIRK1; Inagaki et al., 1995). Again, transient transfection of $uK_{ATP}$-1 in HEK293 cells yielded ATP-sensitive and diazoxide-activated, mildly inward rectifying 70 pS $K^+$ channels. In our research, however, wild-type untransfected HEK293 cells exhibited endogenous ATP-sensitive $K^+$ channels (E. Wischmeyer, unpublished data); thus, the true nature of $K_{ATP}$ channels should be verified in other host cell systems.

What functional role, then, can be attributed to GIRK4? From its structural similarity to GIRK1–3, it may be capable of assembling by itself or in combination with either of the other subfamily members to form functional channel structures, as has been suggested for classic voltage-dependent and, very recently, $K_{IR}$ channels (Kofuji et al., 1995). In the rat atria, where both GIRK1 and GIRK4 appear to be expressed strongly, size-exclusion chromatography defines a hetero-oligomeric complex of 480–520 kDa containing both GIRK1 and CIR proteins in as yet unknown stoichiometry (Krapivinsky et al., 1995). If so, heteromerizations may not be confined to the GIRK subfamily but, rather, may be found across subfamily borders with ROMK- and IRK-type channels as well. The recognition and assembly sequences that restrict subunit heteromerization within the *Shaker* $K^+$ channel subfamily have been confined to two 30- to 60-amino-acid stretches in the T1 region of the N-terminal domain of the protein (Li et al., 1992; Shen and Pfaffinger, 1995). By analogy, it should be possible to define equivalent regions in GIRK-type subunits that control the compatibility with members of other $K_{IR}$ channel subfamilies. It is of particular interest to note that GIRK4/CIR coexpression dramatically increases the current responses of GIRK1/GIRK3 channels severalfold, which is not based on differences in unitary channel conductance alone. If GIRK4 regularly forms heteromeric channels with GIRK1–3 subunits, it is unclear why its cellular expression levels do not match the vastly abundant expression of the other subfamily members in the rat brain. Thus, in addition (or alternative) to the formation of heterotetrameric channel proteins, GIRK4 may play a role in facilitating processing, targeting, or assembly of GIRK-type channel proteins. The fact that GIRK1 protein could be detected by cellular immunoassays in HEK293 cells that lacked functional channel expression (Philipson et al., 1995) indicates that GIRK1 channels in various mammalian cells may be processed inadequately on their way to the membrane. GIRK4 subunits may function to support the process of protein maturation. So far, except for larger basal and receptor-activated macroscopic currents, no other functional alterations that concern gating by G-protein subunits or regulation by intracellular ligands have been detected in coexpression studies. Nevertheless, our observation that GIRK4 and three other members of the GIRK subfamily in the living rat brain are expressed in the same tissue and eventually the same cells (e.g., hippocampal CA3 pyramidal neurons) may have functional consequences. Together with the possibility of alternatively spliced transcripts and the various forms of cell-specific channel regulation via signaling pathways, the multiple possible combinations among GIRK channel subunits may account for the physiological diversity observed in this important family of $K^+$ channels.

## REFERENCES

Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement IV JP, Boyd III AE, González G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA (1995) Cloning of the β cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. Science 268:423–426.

Andrade R, Malenka R, Nicoll R (1986) A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science 234:1261–1265.

Ashcroft SJH, Ashcroft FM (1990) Properties and functions of ATP-sensitive K-channels. Cell Signal 2:197–214.

Ashford MLJ, Bond CT, Blair TA, Adelman JP (1994) Cloning and functional expression of a rat heart $K_{ATP}$ channel. Nature 370:456–459.

Bond CT, Pessia M, Xia XM, Lagrutta A, Kavanaugh MP, Adelman JP (1994) Cloning and expression of a family of inward rectifier potassium channels. Receptors Channels 2:183–191.

Cook DL, Hales CN (1984) Intracellular ATP directly blocks $K^+$ channels in pancreatic β-cells. Nature 311:271–273.

Dascal N, Lim NF, Schreibmayer W, Wang W, Davidson N, Lester HA (1993a) Expression of an atrial G-protein-activated potassium channel in *Xenopus* oocytes. Proc Natl Acad Sci USA 90:6596–6600.

Dascal N, Schreibmayer W, Lim NF, Wang W, Chavkin C, DiMagno L, Labarca C, Kieffer BL, Gaveriaux-Ruff C, Trollinger D, Lester HA, Davidson N (1993b) Atrial G-protein-activated $K^+$ channel: expression cloning and molecular properties. Proc Natl Acad Sci USA 90:10235–10239.

Doupnik CA, Davidson N, Lester HA (1995) The inward rectifier potassium channel family. Curr Opin Neurobiol 5:268–277.

Edwards G, Westeon AH (1990) Potassium channel openers and vascular smooth muscle relaxation. J Pharmacol Exp Ther 48:237–258.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. Pflügers Arch 391:85–100.

Hille B (1992) G protein-coupled mechanisms and nervous signaling. Neuron 9:187–195.

Ho K, Nichols CG, Lederer WJ, Lytton J, Vassilev PM, Kanazirska MV, Hebert SC (1993) Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. Nature 362:31–38.

Inagaki N, Tsuura Y, Namba N, Masuda K, Gonoi T, Horie M, Seino Y, Mizuta M, Seino S (1995) Cloning and functional characterization of a novel ATP-sensitive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. J Biol Chem 270:5691–5694.

Jan LY, Jan YN (1994) Potassium channels and their evolving gates. Nature 371:119–122.

Karschin C, Schreibmayer W, Dascal N, Lester H, Davidson N, Karschin A (1994) Distribution and localization of a G protein-coupled inwardly rectifying $K^+$ channel in the rat. FEBS Lett 348:139–144.

Kobayashi T, Ikeda K, Ichikawa T, Abe S, Togashi S, Kumanishi T (1995) Molecular cloning of a mouse G-protein-activated $K^+$ channel (mGIRK1) and distinct distributions of three GIRK (GIRK1, -2 and -3) mRNAs in mouse brain. Biochem Biophys Res Commun 208:1166–1173.

Kobilka BK, Frielle T, Collins S, Yang-Feng T, Kobilka TS, Francke U, Lefkowitz RJ, Caron MG (1987) An intronless gene encoding a potential member of the family of receptors coupled to guanine nucleotide regulatory proteins. Nature 329:75–79.

Kovoor A, Henry DJ, Chavkin C (1995) Agonist-induced desensitization of the $\mu$ opioid receptor-coupled potassium channel (GIRK1). J Biol Chem 270:589–595.

Koyama H, Morishige KI, Takahashi N, Zanelli JS, Fass DN, Kurachi Y (1994) Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain. FEBS Lett 341:303–307.

Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE (1995) The G-protein-gated atrial $K^+$ channel $I_{KACh}$ is a heteromultimer of two inwardly rectifying $K^+$-channel proteins. Nature 374:135–141.

Kubo Y, Baldwin TJ, Jan YN, Jan LY (1993a) Primary structure and functional expression of a mouse inward rectifier potassium channel. Nature 362:127–133.

Kubo Y, Reuveny E, Slesinger PA, Jan YN, Jan LY (1993b) Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. Nature 364:802–806.

Lesage F, Duprat F, Fink M, Guillemare E, Coppola T, Lazdunski M, Hugnot JP (1994) Cloning provides evidence for a family of inward rectifier and G-protein coupled $K^+$ channels in the brain. FEBS Lett 353:37–42.

Li M, Jan YN, Jan LY (1992) Specification of subunit assembly by the hydrophilic amino-terminal domain of the *Shaker* potassium channel. Science 257:1225–1230.

Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE (1987) The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic $K^+$ channel in the heart. Nature 325:321–326.

Methfessel C, Witzemann V, Takashi T, Mishina M, Numa S, Sakmann B (1986) Patch clamp measurements on *Xenopus* oocytes: currents through endogenous channels and implanted acetylcholine receptor and sodium channels. Pflügers Arch 407:577–588.

Morishige K, Takahashi N, Jahangir A, Yamada M, Koyama H, Zanelli JS, Kurachi Y (1994) Molecular cloning and functional expression of a novel brain-specific inward rectifier potassium channel. FEBS Lett 346:251–256.

Noma A (1983) ATP-regulated $K^+$ channels in cardiac cells. Nature 305:147–148.

North A (1989) Drug receptors and the inhibition of nerve cells. Br J Pharmacol 98:13–28.

Pennington NJ, Kelly JS, Fox AP (1993) Unitary properties of potassium channels activated by 5-HT in acutely isolated rat dorsal raphe neurones. J Physiol (Lond) 469:407–426.

Pennefather PS, Heisler S, MacDonald JF (1988) A potassium conductance contributes to the action of somatostatin-14 to suppress ACTH secretion. Brain Res 444:346–350.

Périer F, Radeke CM, Vandenberg CA (1994) Primary structure and characterization of a small-conductance inwardly rectifying potassium channel from human hippocampus. Proc Natl Acad Sci USA 91:6240–6244.

Pessia M, Bond CT, Kavanaugh MP, Adelman JP (1995) Contributions of the C-terminal domain to gating properties of inward rectifier potassium channels. Neuron 14:1039–1045.

Philipson LH, Kuznetsov A, Toth PT, Murphy JF, Szabo G, Ma GH, Miller RJ (1995) Functional expression of an epitope-tagged G-protein-coupled $K^+$ channel (GIRK1). J Biol Chem 270:14604–14610.

Quast U, Cook NS (1989) Moving together: $K^+$ channel openers and ATP-sensitive $K^+$ channels. Trends Pharmacol Sci 10:431–435.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Shen NV, Pfaffinger PJ (1995) Molecular recognition and assembly sequences involved in the subfamily-specific assembly of voltage-gated $K^+$ channel subunit proteins. Neuron 14:625–633.

Sheng M, Liao YJ, Jan YN, Jan LY (1993) Detection of heteromultimeric $K^+$ channels *in vivo*: potential molecular basis of a presynaptic A-current. Nature 365:72–75.

Takahashi N, Morishige KI, Jahangir A, Yamada M, Findlay I, Koyama H, Kurachi Y (1994) Molecular cloning and functional expression of cDNA encoding a second class of inward rectifier potassium channels in the mouse brain. J Biol Chem 269:23274–23279.

Takao K, Yoshii M, Kanda A, Kokubun S, Nukada T (1994) A region of the muscarinic-gated atrial $K^+$ channel critical for activation by G protein $\beta\gamma$ subunits. Neuron 13:747–755.

Takumi T, Ishii T, Horio Y, Morishige KI, Takahashi N, Yamada M, Yamashita T, Kiyama H, Sohmiya K, Nakanishi S, Kurachi Y (1995) A novel ATP-dependent inward rectifier potassium channel expressed predominantly in glial cells. J Biol Chem 270:16339–16346.

Tang W, Yang XC (1994) Cloning a novel human brain inward rectifier potassium channel and its functional expression in *Xenopus* oocytes. FEBS Lett 348:239–243.

Tempel B, Papazian DM, Schwarz TL, Jan YN, Jan LY (1987) Sequence of a probable potassium channel component encoded at the *Shaker* locus of *Drosophila*. Science 237:770–775.

vanDongen AMJ, Codina J, Olate J, Mattera R, Joho R, Birnbaumer L, Brown AM (1988) Newly identified brain potassium channels gated by the guanine nucleotide binding protein $G_o$. Science 242:1433–1437.

Wang H, Kunkel DD, Martin TM, Schwartzkroin PA, Tempel BL (1993) Heteromultimeric $K^+$ channels in terminal and juxtaparanodal regions of neurons. Nature 365:75–79.

Wischmeyer E, Lentes KU, Karschin A (1995) Physiological and molecular characterization of an IRK-type inward rectifier $K^+$ channel in a tumour mast cell line. Pflügers Arch 429:809–819.

Yatani A, Codina J, Sekura RD, Birnbaumer L, Brown A (1987) Reconstitution of somatostatin and muscarinic receptor mediated stimulation of $K^+$ channels by isolated $G_K$ protein in clonal rat anterior pituitary cell membranes. Mol Endocrinol 1:283–289.

Zhou H, Tate SS, Palmer LG (1994) Primary structure and functional properties of an epithelial K channel. Am J Physiol 266:C809–C824.