Functional and Biochemical Evidence for G-protein-gated Inwardly Rectifying K⁺ (GIRK) Channels Composed of GIRK2 and GIRK3

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G-protein-gated inwardly rectifying K⁺ (GIRK) channels are widely expressed in the brain and are activated by at least eight different neurotransmitters. As K⁺ channels, they drive the transmembrane potential toward $E_K$ when open and thus dampen neuronal excitability. There are four mammalian GIRK subunits (GIRK1–4 or Kir 3.1–4), with GIRK1 being the most unique of the four by possessing a long carboxyl-terminal tail. Early studies suggested that GIRK1 was an integral component of native GIRK channels. However, more recent data indicate that native channels can be either homo- or heterotetrameric complexes composed of several GIRK subunit combinations. The functional implications of subunit composition are poorly understood at present. The purpose of this study was to examine the functional and biochemical properties of GIRK channels formed by the co-assembly of GIRK2 and GIRK3, the most abundant GIRK subunits found in the mammalian brain. To examine the properties of a channel composed of these two subunits, we co-transfected GIRK2 and GIRK3 in CHO-K1 cells and assayed the cells for channel activity by patch clamp. The most significant difference between the putative GIRK2/GIRK3 heteromultimeric channel and GIRK1/GIRKX channels at the single channel level was an ∼5-fold lower sensitivity to activation by Gβγ. Complexes containing only GIRK2 and GIRK3 could be immunoprecipitated from transfected cells and could be purified from native brain tissue. These data indicate that functional GIRK channels composed of GIRK2 and GIRK3 subunits exist in brain.

The G protein-gated class (Kir 3, GIRK)¹ of potassium-selective ion channels are assembled from a small family of subunits. In the nervous system, heart, pancreas, and other tissues, transmitters activate the GIRK channels via an apparently identical Gβγ-linked signal transduction mechanism. The G protein-linked receptor subtypes that activate these channels include muscarinic (M2), γ-aminobutyric acid (GABA$_B$), serotonin (5HT1A), adenosine (P1), somatostatin, enkephalin ($\mu, \kappa, \delta$), $\alpha_2$-adrenergic, and dopamine (D2) receptors. GIRK channels composed of GIRK1 subunits in combination with either GIRK2, GIRK3, or GIRK4 subunits have indistinguishable single channel conductances, open times, and Gβγ sensitivities (1). In contrast, GIRK2, -3, or -4 subunits expressed alone produce spiky, irregular amplitude channels with very brief open times (1–6). GIRK1 subunits alone do not make functional channels but require co-expression of GIRK2, -3, or -4 subunits to achieve membrane localization and yield functional channels that have identical properties to native cardiac I$_{K_{Ca}}$ (7–9). Studies on the GIRK1/GIRK4 channel, I$_{K_{Ca}}$, have indicated that GIRK4 (and presumably GIRK2 and GIRK3) contributes determinants that direct the channels to the membrane (8) as well as cytoplasmic domains that bind Gβγ and are important for channel activation (10–12).

While most brain regions express GIRK1, GIRK2, and GIRK3 mRNAs (13–15), there are regions where only GIRK2 and GIRK3 mRNA transcripts are present or expression of GIRK1 is very low. We combined electrophysiological and biochemical approaches to determine if a GIRK heteromultimer composed of GIRK2 and GIRK3 subunits alone was functional and if such a channel exists in vivo. GIRK2 and GIRK3 subunits had been previously coexpressed in Xenopus oocyte expression studies (16–19), but the presumed GIRK2/GIRK3 currents observed were small (<0.1 μA). In this study, we examine this subunit combination in mammalian cells. We demonstrate that GIRK2/GIRK3 forms a functional channel in heterologous cells and that a GIRK2/GIRK3 complex can be purified from both heterologous expression systems and native brain tissue.

**Experimental Procedures**

**Cell Culture and Transfection—**CHO-K1 cells were grown and maintained in 1:1 Dulbecco’s modified Eagle’s medium/Hamm’s F-12 (Life Technologies, Inc.) with 10% fetal bovine serum at 37 °C under 5% CO2. CHO-K1 cells were transfected using TransIT-R-LT1 (Pan Vera Corp., Madison, WI) in OptiMEM I medium (Life Technologies, Inc.). The pGreen Lantern plasmid (Life Technologies, Inc.) was cotransfected with GIRK-containing pcDNA3 or pcDNA3(-) expression vectors (Invitrogen, Carlsbad, CA) in a 1:4 ratio as a transfection marker for patch clamp experiments. GIRK plasmids were co-transfected in a 1:1 ratio. The GIRK2- and GIRK3-containing plasmids used have been described previously (1, 20). Transfected CHO-K1 cells were detached from their dishes with 5 mM EDTA in PBS, resuspended in growth medium, and transferred to glass coverslips for patch clamp experiments. GIRK plasmids were co-transfected in a 1:1 ratio. The GIRK2- and GIRK3-containing plasmids used have been described previously (1, 20). Transfected CHO-K1 cells were detached from their dishes with 5 mM EDTA in PBS, resuspended in growth medium, and transferred to glass coverslips for patch clamp experiments. COS-7 cells were grown and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum at 37 °C in 5% CO2. COS-7 cells were plated at 2 × 10⁶ cells/100-mm² dish the day before transfection using either LipofectAMINE (Life Technologies) or TransIT-LT1.

**Electrophysiology—**Single channel recordings were made from patches in the inside-out configuration in symmetrical high K⁺ (140 mM KCl, 2 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES, pH 7.2). Patch electrodes were prepared from borosilicate capillary glass (Warner Instrument Corp., Hamden, CT) and polished to a resistance of 5–12

¹ The abbreviations used are: GIRK, G-protein-gated inwardly rectifying K⁺; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-3′-O-(thiotriophosphate); CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholino-propanesulfonic acid; NP$_6$, N is the number of channels in the patch, and $P_o$ is the open probability of the channel.

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GIRK2/GIRK3 Channels

Patches were activated by application of either 40 μM GTP•S (Roche Molecular Biochemicals) or 2–90 mM purified bovine brain Gβγ (2) to the cytoplasmic face of the patch. Currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Inc., Foster City, CA), filtered at 2 kHz by a four-pole low pass Bessel filter, and sampled at 10 kHz.

Records of channels in inside-out patches activated by 40 μM GTP•S were collected at voltages ranging from −100 to −40 mV. Unitary current values from all patches were averaged, and the slope conductance was determined as the best fit to the mean data. Channel open times were determined for 10–50 ms records of inside-out patches activated by 40 μM GTP•S collected at −100 mV. Log analysis (21) was used for determination of channel mean open times (∙)

The concentration dependence of channel activation by Gβγ was examined by adding increasing concentrations of Gβγ to the bath while recording the channel activation. 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 2 nM. Additional Gβγ was added incrementally at 2.5-min intervals until a final concentration of 60 or 90 nM was reached. 2.5 min after the highest Gβγ concentration was attained, 100 μM GTP•S was added to the bath. NP values were determined for the final moment of each Gβγ concentration step. NP values were normalized to the final activity level 1.5 min after the addition of GTP•S for each patch in order to derive relative NP values. Relative NP values from all patches were averaged, and the dose-response curve was determined as the best fit to the mean data.

Immunoprecipitation Assays Using Recombinant GIRK2/GIRK3—Membrane proteins were isolated from single 100-mm² dishes of CHO-K1 cells transfected with GIRK1 plus GIRK2, GIRK1 plus GIRK3, and GIRK2 plus GIRK3. To serve as a control, membrane proteins were also isolated from untransfected cells. The cells were washed twice with PBS before being lifted from the dish with 5 mM EDTA in PBS. The cells were pelleted by centrifugation at 1200 × g for 5 min. Cold hypotonic 5/5/5 lysis buffer (5 mM each of Tris-HCl, EDTA, and EGTA; pH 8.0) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2 μg/ml each of aprotinin, pepstatin, and leupeptin) was added to the cells, which were lysed by ice-cold drawing them through a 23-gauge needle 10 times and a 27-gauge needle four times. Crude membranes were obtained by centrifugation at 120,000 × g for 15 min at 4 °C. The crude membranes were resuspended in 5/5/5 buffer with protease inhibitors, and the proteins were precipitated with 100% (v/v) trichloroacetic acid (Sigma). The precipitated proteins were dissolved in 1× SDS sample buffer and assayed by Western blot with anti-GIRK1 fusion protein antibody αCSh (anti-CSh serum, generated against the carboxy-terminal 156 amino acids of GIRK1) or anti-GIRK1 antibody (Alomone Laboratories, Jerusalem, Israel).

Epitope-tagged GIRK2-Myc and GIRK3-AU1 constructs were transfected into the recombinant coimmunoprecipitation experiments. The GIRK2-Myc has been described previously (20). The transgene was inserted into the Gateway cassette and was kindly provided by Dr. Tanya M. Jelacic. Anti-GIRK2 antibody (Alomone Laboratories) was used at a 1:200 dilution. Samples were incubated for 4 h at 4 °C on a rotator. Bound proteins were eluted with 1× SDS sample buffer at 55 °C for 20 min. The eluted proteins were analyzed by Western blot with anti-GIRK2 antibody (Alomone Laboratories) and rabbit anti-GIRK3 antibody C-312 (Cocalico Biologicals, Inc., Reamstown, PA) against a carboxyl-terminal peptide antigen (SIPSRLDEKVEEEE-FSP) (2). The specificity of the anti-GIRK3 antibody C-312 was confirmed against a panel of recombinant GIRK proteins expressed in COS-7 cells.

RESULTS

Cotransfection of GIRK2 and GIRK3 Yields a Functional Channel in CHO-K1 Cells—In order to examine the properties of a potential channel composed of GIRK2 and GIRK3 subunits, CHO-K1 cells were cotransfected with GIRK2 and GIRK3 and assayed for channel activity by patch clamp. Inside-out patches of transfected cells contained channels that could be activated by application of GTP•S to the cytoplasmic face of the patch and which displayed inward rectification (Fig. 1). The slope conductance of the channel was 31 ± 1.7 pico Siemens (± S.E.; n = 14), smaller than that observed for GIRK1/GIRK2, GIRK1/GIRK3, and GIRK1/GIRK4 channels (35, 39, and 37 picosiemens, respectively) in our previous study (1) but still within the range of conductance values reported for GIRK channels (24). The gΩ at −100 mV for the GIRK2/GIRK3 chan-

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nel was 1.3 ms (n = 11), similar to that for GIRK1/GIRK2, GIRK1/GIRK3, and GIRK1/GIRK4 channels at -80 mV (1.0, 1.1, and 1.2 ms, respectively) (1). These results were surprising, considering that any one of the GIRK2, GIRK3, or GIRK4 subunits expressed alone yields spiky, irregular channels with very brief open times (1, 3–6).

Since activation by Gβγ is a hallmark feature of GIRK channels, the concentration dependence of GIRK2/GIRK3 channel activation was determined. Inside-out patches containing the channel were exposed to increasing concentrations of Gβγ at regularly timed intervals until a final concentration was reached (see Ref. 25). At a set time interval after reaching the final Gβγ concentration, a saturating concentration of GTPγS was added to the bath. The channel activity, quantitated as NPo at each concentration of Gβγ, was normalized to the NPo after the GTPγS step for each patch to derive relative NPo values. Relative NPo values were plotted against log[Gβγ], and a sigmoidal curve was fit to the mean data. Channel activity increased in response to increasing Gβγ, with a half-maximal concentration of 53 nM (n = 4 patches). GIRK1/GIRK3 data are replotted from Ref. 1 for comparison.

two dose-response curves, but GIRK1/GIRK3 is significantly more sensitive to Gβγ than GIRK2/GIRK3; F (1, 25) = 8.77, p = 0.007.

FIG. 1. Inwardly rectifying channel currents are seen when GIRK2 and GIRK3 are co-expressed in CHO-K1 cells. CHO-K1 cells expressing GIRK2 and GIRK3 were analyzed by patch clamp in the inside-out configuration in symmetrical 140 mM K+ Channels were activated by application of 40 μM GTPγS to the cytoplasmic face of the patch. A, records were made at transmembrane potentials both positive and negative to the reversal potential of 0 mV to verify inward rectification. B, channel currents were recorded at various transmembrane potentials. Current-voltage relation reveals a unitary conductance of 31 picosiemens (pS) (mean ± S.E.; n = 14 patches). C, open time distribution at -100 mV is shown with a dwell time histogram.

FIG. 2. Dose-response curve of GIRK2/GIRK3 channels to Gβγ. Increasing amounts of Gβγ were applied to the cytoplasmic face of inside-out patches expressing GIRK2/GIRK3 at set time intervals (see “Experimental Procedures”). At the end of each experiment, 100 μM GTPγS was applied. The activity level for each concentration of Gβγ was quantitated as NPo, and normalized to the NPo for the GTPγS step for each patch to derive relative NPo values. Relative NPo values were plotted against log[Gβγ], and a sigmoidal curve was fit to the mean data. Channel activity increased in response to increasing Gβγ, with a half-maximal concentration of 53 nM (n = 4 patches). GIRK1/GIRK3 data are replotted from Ref. 1 for comparison.

FIG. 3. Cotransfection of GIRK2 and GIRK3 does not induce expression of GIRK1 in CHO-K1 cells. CHO-K1 cells were co-transfected with GIRK1 plus GIRK2, GIRK1 plus GIRK3, or GIRK2 plus GIRK3. Control cells were not transfected. Membrane proteins were harvested from all cells and analyzed for GIRK1 by Western blot. Only cells transfected with the GIRK1 construct expressed GIRK1 protein.
GIRK1 protein could be detected only in cells that had been transfected with GIRK1 cDNA (Fig. 3).

**GIRK2 and GIRK3 Form a Complex in Transfected Cells**—To examine whether or not cotransfected GIRK2 and GIRK3 subunits were able to form heteromultimeric complexes, we coimmunoprecipitated the GIRK channel proteins from COS-7 cells transfected with epitope-tagged GIRK subunits. After cotransfection with the GIRK2-Myc and GIRK3-AU1 constructs, membrane proteins from COS-7 cells were harvested, and ascites fluid for monoclonal AU1 antibodies was used to immunoprecipitate GIRK3-AU1 and any associated proteins. These proteins were eluted and transferred to a membrane for Western blot analysis with subunit-specific anti-GIRK2 and anti-GIRK3 antibodies. The specific recognition of their respective GIRK proteins by these antibodies can be seen in Fig. 4, A and B. Analysis of the eluted proteins by Western blot demonstrated the presence of both GIRK3 and GIRK2 in the AU1 ascites pellet (Fig. 4C). These coimmunoprecipitation data strongly support the notion that GIRK2 and GIRK3 subunits can combine to form a heteromultimeric complex in transfected mammalian cells.

**GIRK2/GIRK3 Channels Are Present in Native Brain**—To test whether heteromultimeric complexes of GIRK2 and GIRK3 subunits might exist in vivo, we performed coimmunoprecipitation experiments using mouse brain as the tissue source. Membranes solubilized in 1% Triton X-100 buffer were prepared from wild-type and GIRK3 knockout (control) mouse brains. Unlike the heterologous expression system, the native system is complicated by the presence of GIRK1. A straightforward GIRK2/GIRK3 coimmunoprecipitation experiment would have ambiguous results because it would be uncertain whether or not the complex containing GIRK2 and GIRK3 also contained GIRK1. To avoid this ambiguity, the solubilized membranes were first immunodepleted of GIRK1 by two rounds of GIRK1 immunoprecipitation before immunoprecipitation of GIRK2 and GIRK3.

After the second round of GIRK1 immunodepletion, small supernatant samples were tested for GIRK1 by Western analysis. As Fig. 5A shows, GIRK1 protein was successfully removed, leaving a GIRK1-free protein pool from which we could immunoprecipitate GIRK2 and GIRK3. The final immunoprecipitates and control supernatants were analyzed by Western blot for GIRK2 and GIRK3 coimmunoprecipitation.

Fig. 5B shows the final immunoprecipitates and control su-
permanants probed with anti-GIRK2 antibody. The GIRK2 signal from native brain membranes consistently appeared as a doublet, suggesting that at least two GIRK2 isoforms are present (26, 27). GIRK2 protein was recovered from the control supernatant and both the anti-GIRK2 and anti-GIRK3 immunoprecipitates of the wild-type samples but was recovered only from the control supernatant and the anti-GIRK2 immunoprecipitate of the GIRK3 knockout samples. The presence of GIRK2 in the anti-GIRK3 wild-type immunoprecipitate together with its absence from the anti-GIRK3 knockout immunoprecipitate indicates that GIRK2 was pulled down by the anti-GIRK3 antibody most likely because it was complexed with GIRK3. Fig. 5c shows the final immunoprecipitates and control supernatants probed with an anti-GIRK3 antibody. GIRK3 was recovered from the control supernatant and both the anti-GIRK2 and anti-GIRK3 immunoprecipitates of the wild-type samples. As expected, no GIRK3 signal was seen for the GIRK3 knockout samples. The presence of GIRK3 in the anti-GIRK2 wild-type immunoprecipitate reinforces the evidence of coimmunoprecipitation seen with the anti-GIRK3 antibody. These results demonstrate that complexes containing GIRK2 and GIRK3 without GIRK1 can be immunoprecipitated from native brain.

**DISCUSSION**

We have demonstrated that GIRK2/GIRK3 channels are functional, exist in native brain tissue, and are less sensitive to Gβγ than GIRK1-containing channels. When GIRK2, GIRK3, or GIRK4 subunits are transfected alone, each gives rise to “spiky” channels with very brief open times (<0.5 ms) and variable single channel conductance (20–40 picosiemens) (1, 3, 5, 6). Co-transfecting either subunit with GIRK1 yields a heteromultimeric channel with characteristics like the prototypi- cal GIRK channel, IKACh. The uniqueness of GIRK1 has attracted a series of hypotheses to account for its long carboxy- tail region of GIRK4 are critical for activation of IKACh (10). The similarity of this region (e.g. C216) in GIRK2 and GIRK3 probably accounts in part for the similar responses of the GIRK1/GIRK4 and GIRK1/GIRK3 channels to Gβγ (1). However, the same study and others have demonstrated that GIRK1 also binds Gβγ (11, 12, 28). Thus, Gβγ binding to GIRK1 may have a potentiating effect, or perhaps GIRK1 and GIRK4 both contribute to the binding site for Gβγ, and GIRK1’s contribution may be to increase the affinity for Gβγ. Alternatively, GIRK1 may contribute to or affect the binding site for a modulating molecule such as inositol 1,4,5-trisphosphate, which has been shown to play an important role in the activation of IKACh by Gβγ and by intracellular Na+ (29–33).

The lower sensitivity of GIRK2/GIRK3 channels to activation by Gβγ may partly explain why the GIRK currents in *Xenopus* oocytes co-injected with cRNAs for these subunits are so small (16–19). Additionally, GIRK3 apparently expresses at low levels in oocytes compared with the other GIRK subunits (16–19, 34). The combination of poor expression of the channel with its decreased sensitivity to Gβγ may account for the small peak currents observed for GIRK2/GIRK3 in *Xenopus* oocytes. In contrast, GIRK3 and GIRK2/GIRK3 express well in mammalian cells.

In addition to providing enhanced Gβγ sensitivity, GIRK1 may also provide a second localization mechanism via its unique carboxyl tail. Additional diversity comes from the fact that GIRK3 and the long form of GIRK2 both have potential PDZ-binding motifs at their carboxyl termini. The variety of GIRK channel combinations may allow for specificity of localization of channels in the neuron, at pre- or postsynaptic sites for example, or for linking of channel subtypes to specific neurotransmitter receptors.

Most regions in the brain have the potential to produce GIRK2/GIRK3 channels because they express GIRK1, GIRK2, and GIRK3 mRNAs (13–15). The expression of GIRK1 does not preclude the formation of complexes that do not contain GIRK1, and cells may produce more than one type of GIRK channel. This is clearly illustrated by the presence of GIRK4 homomeric complexes in atrial cells expressing IKACh (35). It is possible that a neuron that produces GIRK1/GIRK2 and GIRK1/GIRK3 channels may also produce GIRK2/GIRK3 channels. GIRK2/GIRK3 channels have a different sensitivity to activation by Gβγ and may be localized differently than channels containing GIRK1. Thus, there may be regions within neurons where GIRK2/GIRK3 channels are more common than GIRK1/GIRK2 or GIRK1/GIRK3 channel complexes.

In summary, we have demonstrated the existence in brain of a heteromultimeric GIRK channel composed of GIRK2 and GIRK3 subunits. These channels have a similar open time to the GIRK1/GIRKx channels but have a slightly smaller conductance and less sensitivity to activation by Gβγ. The discovery of this channel, with its lesser sensitivity to Gβγ and hence
lower threshold of activation, along with its potential for differential localization from the GIRK1/GIRKx channels, broadens the functional scope of the GIRK family of channels.

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