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Evidence that neuronal G-protein-gated inwardly rectifying K^+ channels are activated by $G\beta\gamma$ subunits and function as heteromultimers

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ABSTRACT **Guanine nucleotide-binding proteins (G proteins) activate K^+ conductances in cardiac atrial cells to slow heart rate and in neurons to decrease excitability. cDNAs encoding three isoforms of a G-protein-coupled, inwardly rectifying K^+ channel (GIRK) have recently been cloned from cardiac (GIRK1/Kir 3.1) and brain cDNA libraries (GIRK2/Kir 3.2 and GIRK3/Kir 3.3). Here we report that GIRK2 but not GIRK3 can be activated by G protein subunits $G\beta_1$ and $G\gamma_2$ in *Xenopus* oocytes. Furthermore, when either GIRK3 or GIRK2 was coexpressed with GIRK1 and activated either by muscarinic receptors or by $G\beta\gamma$ subunits, G-protein-mediated inward currents were increased by 5- to 40-fold. The single-channel conductance for GIRK1 plus GIRK2 coexpression was intermediate between those for GIRK1 alone and for GIRK2 alone, and voltage-jump kinetics for the coexpressed channels displayed new kinetic properties. On the other hand, coexpression of GIRK3 with GIRK2 suppressed the GIRK2 alone response. These studies suggest that formation of heteromultimers involving the several GIRKs is an important mechanism for generating diversity in expression level and function of neurotransmitter-coupled, inward rectifier K^+ channels.**

Binding of neurotransmitters or hormones to seven-helix receptors regulates the activity of many ion-selective channels (1). In cardiac atrium, stimulation of muscarinic receptors leads to the opening of inwardly rectifying K^+ channels (2, 3) with a much faster time course (100–500 ms) (4, 5) than for most effects of seven-helix receptors on channels. The atrial pathway has the features expected for the involvement of guanine nucleotide-binding proteins (G proteins) of the G_i or G_o class (2, 3) and proceeds without the mandatory mediation of a diffusible, cytoplasmic second messenger (3, 6). The atrial channel is also activated by exogenously applied G protein $G\beta\gamma$ subunits to the cytoplasmic face of excised patches (7–9), suggesting that direct channel–G protein association causes channel activation (10).

While channel activation by this membrane-delimited pathway was first described and characterized in atrial cells, it is now clear that similar mechanisms are widespread in neurons as well, underlying inhibitory actions of several nonpeptide and peptide neurotransmitters (11). A pertussis-toxin-sensitive, neurotransmitter-activated, inwardly rectifying K^+ conductance is found in hippocampus (12, 13), dorsal raphe (14, 15), and locus coeruleus (16, 17).

Recently, the muscarinic channel from cardiac atrium has been cloned and named GIRK1 (for G-protein coupled, inwardly rectifying K^+ channel 1), KGA, or Kir3.1 (18–20). GIRK1 expression in *Xenopus* oocytes mimics the main features of the native atrial channel, including activation by the m2 type muscarinic receptor (m2R), strong inward rectification of currents, and maintained activation when the channel is coexpressed with G protein subunits $G\beta_1$ and $G\gamma_2$ (18, 21). Two additional clones have been isolated by low-stringency screening of a mouse brain cDNA library with a rat GIRK1 probe (22). These clones were named mbGIRK2 and mbGIRK3 [Kir3.2 and Kir3.3, respectively (20)] because they show slightly greater sequence similarity to GIRK1 than to other cloned channels. δ-opioid receptors couple functionally to GIRK2 but not GIRK3 channels (22).

Although present evidence suggests that the cardiac channel GIRK1 is activated by $G\beta\gamma$ subunits (7–10), it has been claimed that $G\alpha_o$ subunits activate inwardly rectifying K^+ channels in excised patches from hippocampal cells (23), raising the question whether the neuronal channels GIRK2 and GIRK3 are activated by G protein $G\alpha$ subunits and/or by $\beta\gamma$ subunits. Furthermore, given that GIRK1, GIRK2, and GIRK3 mRNAs are present in brain (18, 19, 22, 24), we wished to know whether these different GIRKs are able to associate with each other to form functional multisubunit, heteromultimeric channels. Here, we show that GIRK2 but not GIRK3 can be directly activated on coexpression of G protein subunits $G\beta_1$ and $G\gamma_2$ in *Xenopus* oocytes. Most important, we find that coinjection of GIRK1 with GIRK2 or GIRK3 cRNA gives a much large G-protein-induced current than the responses for each cRNA expressed individually but that, surprisingly, coinjection of GIRK3 with GIRK2 mRNA suppresses the G protein response of the latter. These results demonstrate the formation of functional heteromultimeric channels in *Xenopus* oocytes.

MATERIALS AND METHODS

Plasmids and DNAs. To isolate GIRK2 and GIRK3 from mouse brain RNA, we designed oligonucleotides that anneal to the first assigned methionine codon and the assigned stop codon of mouse GIRK2 and GIRK3 (22). GIRK-specific sequences were coupled to a 5′-end untranslated sequence from alfalfa mosaic virus (25, 26) and a T7 RNA polymerase recognition site to confer an optimal translational initiation site and allow RNA synthesis *in vitro* (cRNA). At the 3′ end, a poly(A) tail was added to confer RNA stability. Total RNA from adult mouse brain (purchased from Clontech) was used as template for cDNA synthesis (27). PCR was conducted in a thermal cycler (Perkin–Elmer) by using Vent Polymerase (New England Biolabs), which minimizes misincorporations during the amplification step. GIRK2 and GIRK3 PCR-derived sequences were then inserted into the pNoTA vector (5 Prime → 3 Primer, Inc.) for subsequent sequence analysis.

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Abbreviations: G protein, guanine nucleotide-binding protein; GIRK, G-protein-coupled, inwardly rectifying K^+ channel; ACh, acetylcholine; $I_{K,ACh}$ and I_K, inward currents induced by ACh and high $[K^+]$, respectively; m2R, m2 type muscarinic receptor; E_K, K^+ equilibrium potential.

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GIRK1 cloned from cardiac atrium (19) and inserted originally in pBluescript (Stratagene) was transferred to the vector pMXT (gift from J. Yang; University of Texas, Dallas) in which the cloning site is flanked by 5′ and 3′ untranslated sequences from *Xenopus* globin. G protein subunits $G\beta_1$ and $G\gamma_2$ cDNAs were in the pFrogy vector (gift from L. Jan, University of California, San Francisco), as described (28). The m2R cDNA (gift from E. Peralta, Harvard University) was in the pGEM3 vector (Promega).

RNA Synthesis and Oocyte Injections. GIRK2 and GIRK3 cRNAs were synthesized directly from gel-isolated PCR products, while the remaining cRNAs were synthesized from linearized plasmid DNAs. cRNAs were dissolved in sterile water and injected into stage V or VI *Xenopus* oocytes as described (29). Oocytes were maintained in ND96 solution (96 mM NaCl/2 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.5 with NaOH/2.5 mM sodium pyruvate/0.5 mM theophylline/50 mg of gentamycin per liter. Oocytes were assayed 2–5 days after injection.

Electrophysiology. Whole-cell currents from oocytes were measured by using an Axoclamp 2A or Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) in the two-electrode, voltage-clamp configuration. Current and voltage electrodes were filled with 3 M KCl to yield resistances ranging from 0.5 to 1.5 MΩ. Recordings were started in an external solution (0 K^+) containing 98 mM NaCl, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.3. In high-K^+-containing solutions, the NaCl was replaced either completely, by 98 mM KCl (98 K^+), or partially, by 20 mM KCl (20 K^+).

Cell-attached recordings of single channels were recorded from *Xenopus* oocytes as described (30). Pipette solutions contained 150 mM KCl, 1 mM $CaCl_2$, and 5 mM Hepes, pH 7.3 with KOH. Bath solution contained 150 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 5 mM Hepes, pH 7.3 with KOH. For single-channel analysis, the current traces were filtered at 2 kHz and sampled at 10 kHz. Current amplitude histograms and open-time durations were obtained by using FETCHAN and pSTAT from pCLAMP 6.0 (Axon Instruments). All recordings were performed at room temperature (≈22°C).

RESULTS

GIRK1 is abundantly expressed in cardiac atrium and brain (18, 19, 24, 31). When heterologously expressed in *Xenopus* oocytes, GIRK1 induces strong inwardly rectifying K^+ currents either with m2R activation (19) or with coexpression of G protein subunits $G\beta_1$ or $G\gamma_2$ (21, 28).

Evidence from Activation via Muscarinic Receptors. Because expression of GIRK2 and GIRK1 was consistently smaller than for other channels expressed in oocytes (see *Discussion*), we have examined the effects of GIRK1 and GIRK2 coexpression under conditions of maximal m2R activation [1 μM acetylcholine (ACh)]. With the coinjection of GIRK1 and GIRK2 (GIRK1 + 2) cRNAs (0.5 ng each per oocyte), exposure to ACh in these oocytes led to development of large inward currents when the voltage was jumped from 0 mV to more negative values (Fig. 1). By contrast, in oocytes injected with GIRK2 or GIRK1 cRNAs (1 ng), receptor-activated currents were much smaller in amplitude, comparable to levels reported previously (18, 19, 22). On average, GIRK1 + 2 currents [inward currents induced by ACh ($I_{K,ACh}$) = −6.4 ± 0.8 μA; mean ± SEM; n = 5] were about 9-fold larger than GIRK2 currents ($I_{K,ACh}$ = −698 ± 98 nA; n = 7) and 17-fold larger than GIRK1 currents ($I_{K,ACh}$ = −365 ± 45 nA; n = 5).

GIRK1 expressed in oocytes shows distinctive gating kinetics, including slow phases of activation (several hundred ms) during a jump from 0 mV to more negative potentials (32) (Fig. 1). For a similar voltage jump, GIRK2 expressed in oocytes showed kinetics that more closely resemble other strong inward rectifiers, with a prominent phase of inactivation. We believe that these differences are governed at least partially by differences in the sequence of the P region (33). The coexpressed subunits showed a slow phase of activation (Fig. 1), but the time course of this activation cannot be explained as a

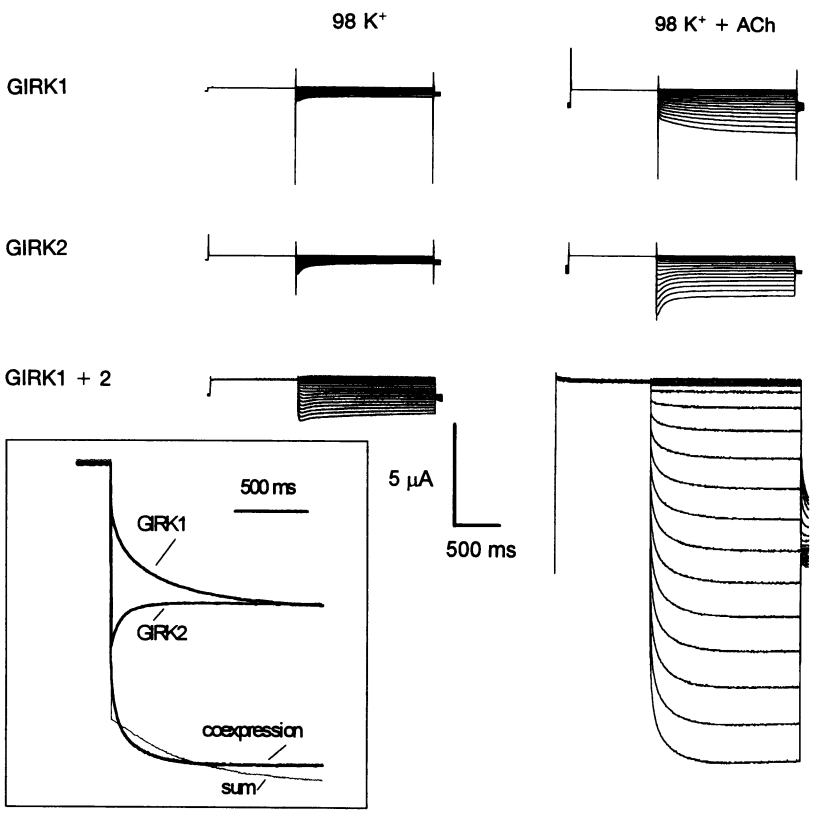

FIG. 1. Enhancement of m2R-evoked responses by GIRK2 and GIRK1 coexpression. *Xenopus* oocytes were injected with m2R (1 ng per oocyte) cRNA, as well as GIRK1 (1 ng) cRNA, GIRK2 cRNA (1 ng), or GIRK1 plus GIRK2 cRNAs (0.5 ng each). Whole-cell currents were recorded 2 days postinjection in 98 K^+ bath solution in the presence and absence of 1 μM ACh (saturating concentration for the activation of m2R). Membrane potential was held at 0 mV for 1 sec, then stepped to test potentials varying from −140 to +20 mV. Data are typical of six oocytes at each condition. (*Inset*) Further analysis of the traces superimposed for the jump to −140 mV. Current traces are ACh-sensitive currents obtained by subtracting traces on the left from those on the right. Traces for GIRK1 and GIRK2 are expanded to roughly half the amplitude of the trace for coexpression, then added (thin line). The summed trace is a poor fit to the actual data, suggesting that the waveforms for the coexpressed channels cannot be expressed as a linear sum of the traces for GIRK1 alone and GIRK2 alone.

simple weighted sum of the waveforms for GIRK1 alone and GIRK2 alone (Fig. 1*Inset*). Thus, the relaxations for jumps to voltages between −60 and −140 mV were well described by two exponential components with nearly voltage-independent time constants; at −80 mV the time constant of the slower component was 213 ± 12 ms (n = 4), or less than half that for GIRK1 (32). These kinetic differences indicate molecular interactions between the GIRK1 and GIRK2 channels.

Evidence from Activation via βγ Subunits. The large enhancement of the agonist-evoked currents for coexpressed channels might be explained by effects on any component in the receptor–channel signaling pathway, including receptors, endogenous G proteins, or the channels themselves. To discriminate among these possibilities, we uncoupled the receptor from the channel by overexpression of G protein subunits $G\beta_1$ and $G\gamma_2$. Importantly, cells expressing $G\beta_1\gamma_2$ and GIRK2 showed persistent inwardly rectifying currents at amplitudes comparable or larger to those observed for m2R activation of GIRK2 (current–voltage relationships for representative oocytes are shown in Fig. 2*A*). Therefore, GIRK2, like GIRK1, is activated by $G\beta\gamma$ subunits.

Cells coexpressing GIRK2 and GIRK1 (GIRK1 + 2) responded to high-K^+ solution with much larger currents than did cells expressing only GIRK1 or GIRK2, consistent with the results obtained with m2R activation. On average, GIRK1 + 2 currents (I_K = −4.3 ± 1 μA; n = 7) were 14 times larger than GIRK2 currents (I_K = −305 ± 56 nA; n = 7) and 40 times larger than GIRK1 currents (I_K = −104 ± 10 nA; n = 6) (Fig. 2*B*). Thus, these experiments demonstrate that the large mutual potentiating effect of GIRK1 + 2 is independent of the method of G protein activation and argue that the effects are on the channels themselves.

Evidence from Single-Channel Conductance and Kinetics. An increase in the number of channels or modification of the intrinsic channel properties might account for this potentiation. Single-channel recordings of GIRK1 + 2 in combination or of GIRK2 alone were made to test these possibilities. In oocytes coinjected with cRNAs for GIRK2, $G\beta_1$, and $G\gamma_2$, single-channel currents in the cell-attached configuration displayed features consistent with macroscopic measurements—i.e., with hyperpolarization the unit conductance increased. No outward currents were detected at membrane potentials positive to the K^+ equilibrium potential (E_K; ≈0 mV) (Fig. 3*A*). These channel openings had a mean slope conductance of 30 ± 2 pS (n = 4) over the range from −40 to −100 mV, significantly smaller than the value of 39 pS for GIRK1 alone (19), and showed bursts of flickery activity. Mean open-time distribution could be described by a fast component of 0.1 ms (35% of the total number of events) and a main slower component of 0.5 ms (65% of the total number of events) (Fig. 3*B*).

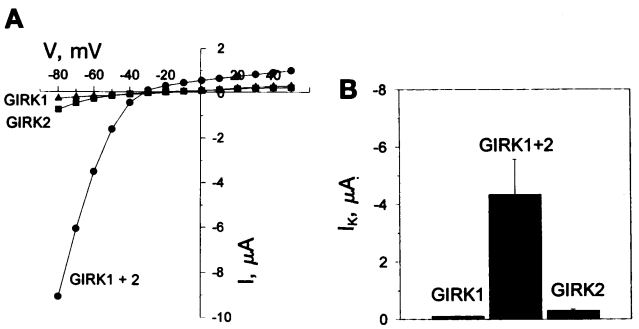

FIG. 2. GIRK2 enhances $G\beta_1\gamma_2$ evoked currents when coexpressed with GIRK1. (*A*) Current–voltage relationship for representative oocytes injected with cRNA for G protein subunits $G\beta_1$ and $G\gamma_2$ (5 ng each), as well as for GIRK2 (1 ng), GIRK1 (1 ng), or GIRK1 plus GIRK2 (0.5 ng each). Currents were measured 2 days postinjection. Bath solution contained 20 mM K^+. Note the large increase of inward currents upon coexpression of GIRK1 and GIRK2. (*B*) Average inward K^+ currents in the presence of high [K^+] (I_K) were obtained by subtracting traces in 0 K^+ from those in 20 K^+ at −80 mV. Data are from four to six oocytes for each condition.

The combination of GIRK1 + 2 produced unitary currents with strong inward rectification and a mean slope conductance of 35 ± 3 pS (n = 4), intermediate between the values for GIRK1 alone and GIRK2 alone (Fig. 3*A*). In addition, GIRK1 + 2 channels displayed markedly longer openings than GIRK2 channels (Fig. 3*B*). Recordings from GIRK1 + 2 channels showed a 7-fold increase in the major component of open-time duration (3.5 ms; 71% of the total number of events; there was also a smaller component of 0.5 ms; 29% of the total number of events) compared to GIRK2. Qualitatively, the GIRK1 + 2 patch recordings are rather similar to those observed for GIRK1 in terms of mean open time (see ref. 18), although the typically low expression levels for GIRK1 alone vitiate systematic comparisons. While we did not accumulate systematic data, it is our strong impression that the probability of finding patches with multiple channels was greatly increased for coinjected oocytes. At present, it is not known whether this increase, like the increase in macroscopic currents on GIRK1 + 2 coexpression, is solely explained by the increase in open-time duration or whether the enhanced currents also arise from an increase in the number of openings, either because each channel opens more often or because there are more functional channels.

Evidence from GIRK3 Coexpression with GIRK1 or GIRK2. We also tested the effects of GIRK3 coexpression with GIRK1 or GIRK2. Injection of GIRK3, $G\beta_1$, and $G\gamma_2$ cRNAs did not activate inward currents in high-K^+-containing solution (data not shown); nor were currents activated by opioid receptors (22). Nevertheless, GIRK3 had profound effects when coexpressed with GIRK1 (Fig. 4*A*). Coexpression of GIRK1 and GIRK3 (GIRK1 + 3) resulted in 7-fold larger I_K currents (I_K = −3.4 ± 0.5 μA; n = 6) than for GIRK1 alone (I_K = −481 ± 43 nA; n = 6). Such augmentation was seen for each of several batches of oocytes and cRNAs and on activation of the m2R pathway (data not shown). Thus, these results demonstrate that GIRK3, despite not being directly activated by G protein subunits $G\beta_1$ and $G\gamma_2$, can interact with and increase G-protein-mediated responses of GIRK1.

Surprisingly, GIRK3 had a suppressing effect when coexpressed with GIRK2 (Fig. 4*B*). $G\beta\gamma$-induced, inwardly rectifying currents in oocytes coinjected with GIRK2 and GIRK3 cRNAs (0.5 ng each per oocyte) (I_K = −129 ± 22 nA; n = 7) were much smaller than in oocytes injected with GIRK2 cRNA alone (1 ng per oocyte) (I_K = −5.1 ± 0.6 μA; n = 9). This dominant-negative effect of GIRK3 on GIRK2 currents was also observed when we coexpressed m2R instead of G protein subunits $G\beta_1$ and $G\gamma_2$ (data not shown).

DISCUSSION

Expression of GIRK1 isolated from atria in *Xenopus* oocytes induced currents with many of expected properties, including activation by G_i-linked receptors (18, 19) and by G protein subunits $G\beta\gamma$ (21, 28). However, some anomalies were noted during the course of our studies with GIRK1. First, current amplitudes were not much larger than those observed with expression from tissue poly(A)$^+$ RNA (compare refs. 19 and 34). Maneuvers designed to raise the expression levels, such as increasing the amount of injected GIRK1 cRNA or modifying untranslated regions, were generally ineffective. Second, heterologous expression of GIRK1 has been successful only in *Xenopus* oocytes. We and others have attempted to express GIRK1 in mammalian cells by plasmid-mediated transient transfection or vaccinia virus infection. We have observed in all cases the absence of GIRK1-like currents in mammalian cells, despite the presence of large amounts of infection/transfection-induced GIRK1 mRNA in these cells (Jun Li, personal communication). Such

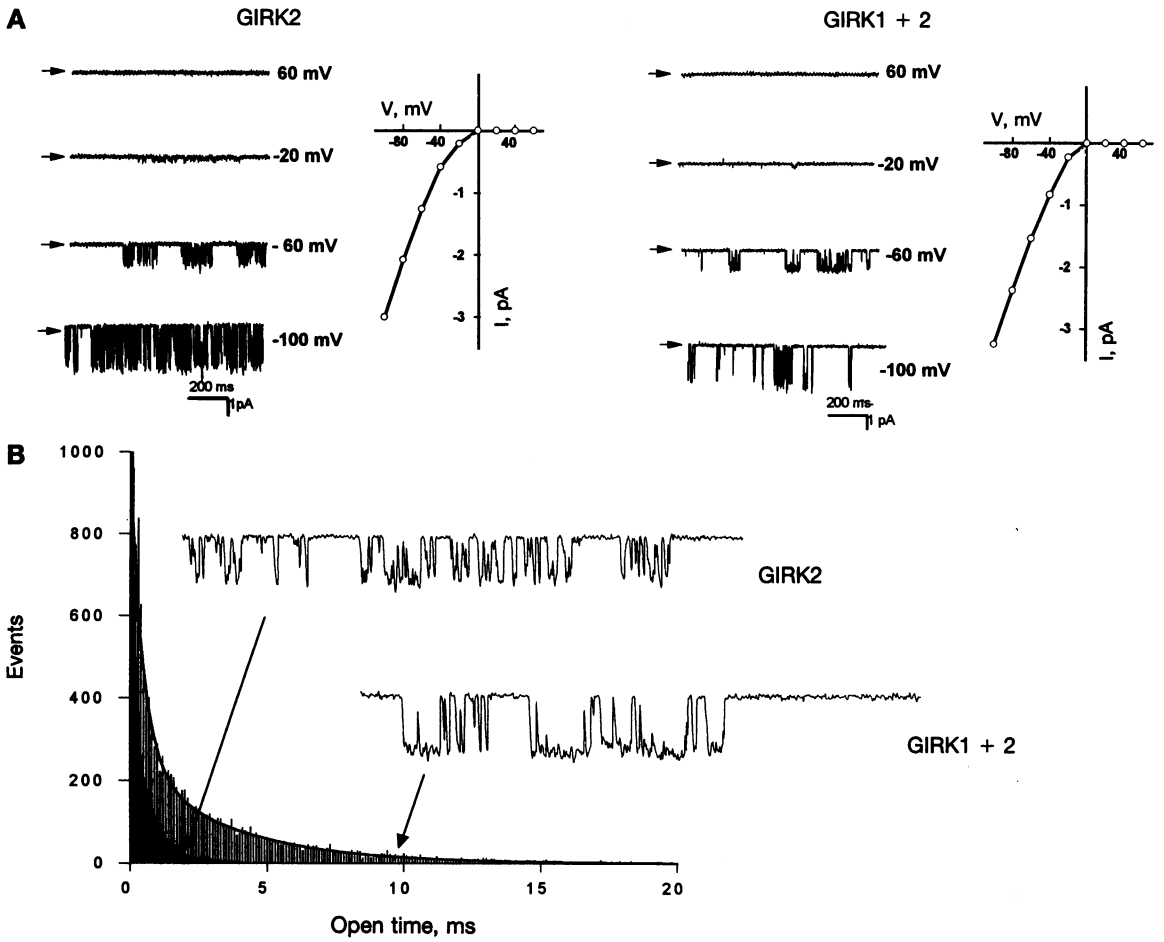

FIG. 3. Single-channel recordings from oocytes expressing GIRK1 + 2 or GIRK2 channels. (*A*) Cell-attached patch recordings from an oocyte coinjected with $G\beta_1$ and $G\gamma_2$ cRNAs, as well as GIRK2 cRNA or GIRK1 and GIRK2 cRNAs. Membrane potential was varied from −100 to +60 mV. Arrows indicate the zero current level. Current amplitudes were measured from all-points histogram data at each membrane potential. (*B*) Open-time distributions for GIRK2 + 1 and GIRK2 channels at −80 mV. Current recordings were low-pass filtered at 2 kHz.

idiosyncrasies of expression levels and cellular environment suggested the involvement of accessory subunits for efficient assembly of the G-protein coupled, inwardly rectifying K^+ channels or coupling with G proteins.

Here we have shown that GIRK2 and GIRK3, when coexpressed with GIRK1, are able to interact and produce large (5- to 40-fold) enhancement of the G-protein-evoked currents. Further analysis of GIRK1 + 2 expression shows that the voltage-jump kinetics cannot be explained as a simple weighted sum of the traces for the individual channels, and the single-channel conductances are intermediate between those for the individual channels. These data show that GIRK1 with GIRK2 and probably GIRK1 with GIRK3 can form heteromultimeric channels leading to larger G-protein-activated currents with distinct physiological properties.

Heteromultimerization among distinct but highly similar subunits is described for other channels, such as the amiloride-sensitive sodium channel (35) and the retinal and olfactory cyclic nucleotide-gated cation channels (36, 37). We cannot be certain at this moment how coexpression of GIRK1 and GIRK2 or GIRK3 results in such large potentiation of the G-protein-evoked responses. Single-channel recordings from the GIRK2 and GIRK1 combination show that individual channel proteins remain open several times longer; but there may be additional factors, such as more frequent openings or an increased number of functionally competent channels.

While this manuscript was in preparation, it was reported that in atrial cells (38), the GIRK1 polypeptide associates with another member of the inwardly rectifying K^+ channel superfamily, CIR, demonstrated by immunoprecipitation of the heteromultimeric channels from atrial membranes by GIRK1-specific antibodies. CIR is identical in sequence to the channel previously identified as K_{ATP} (39). However, the CIR channel has the features expected from a G-protein-coupled channel: it is activated by nonhydrolyzable analogs of GTP and by G protein subunits $G\beta\gamma$ (38). Furthermore, GIRK1 and CIR coexpression results in large potentiation of the G-protein-gated currents in *Xenopus* oocytes (38). Such findings are consistent with the hypothesis that in the atrial cells, the G-protein-gated channel is a heteromultimer of at least two distinct, inwardly rectifying K^+ channels (GIRK1 and CIR). We speculate, as did Krapivinski *et al.* (38), that when GIRK1 alone is expressed in oocytes, functional channels are heteromers of GIRK1 with a GIRK-like protein endogeneous to oocytes.

Until immunoprecipitation experiments are done with brain membranes, we can only speculate that GIRK2 also coassembles with GIRK1 in neuronal cells, but this seems likely given the strong similarities between CIR (38) and GIRK2. (*i*) CIR is more closely related to GIRK2 than to any other known protein (20, 38). (*ii*) Both CIR and GIRK2 can be directly activated by $G\beta\gamma$ subunits (38). (*iii*) Homomeric CIR and GIRK2 unitary currents display similarly brief, single-channel openings. (*iv*) Both CIR and GIRK2 increase the magnitude of evoked currents many fold upon coexpression with GIRK1.

Receptor-activated, inwardly rectifying K^+ conductances are found extensively in the brain and may underlie the postsynaptic inhibitory action of many neurotransmitters and neuropeptides (11). Which GIRK subunits account for these conductances?

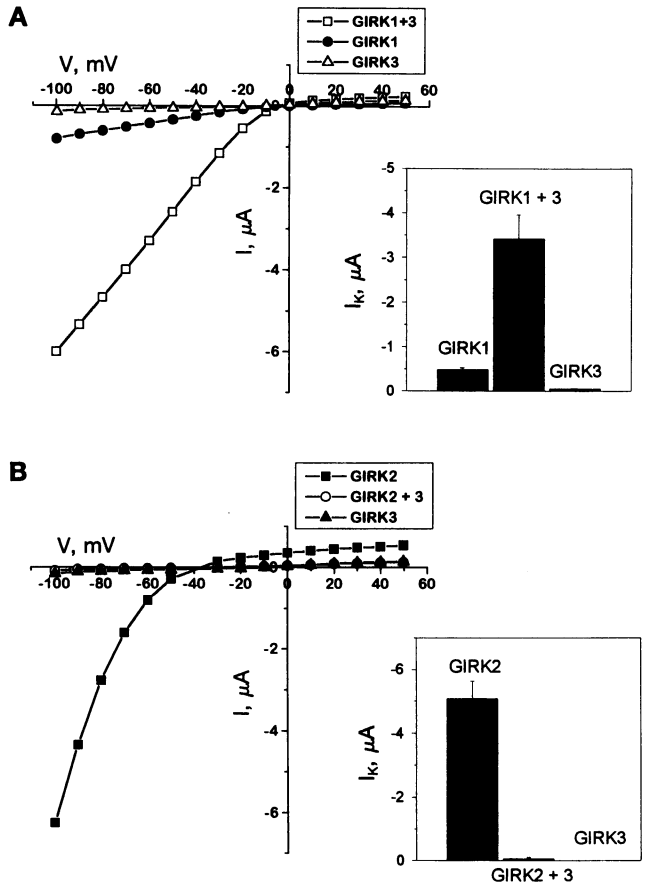

FIG. 4. GIRK3 has opposite effects on G-protein-evoked currents when coexpressed with GIRK1 or GIRK2. (*A*) Current–voltage relationships for typical oocytes injected with $G\beta_1$ and $G\gamma_2$ cRNAs (5 ng each), as well as GIRK3 cRNA (1 ng), GIRK1 cRNA (1 ng), or GIRK1 and GIRK3 cRNAs (0.5 ng each). Bath solution contained 98 mM K^+. Note the large increase of inward currents upon coexpression of GIRK1 and GIRK3. (*Inset*) Average K^+ currents at −80 mV. (*B*) Current–voltage relationships for typical oocytes injected with $G\beta_1$ and $G\gamma_2$ cRNAs, as well as either GIRK2 cRNA (1 ng), GIRK3 cRNA (1 ng), or GIRK3 and GIRK2 cRNA (0.5 ng each). (*Inset*) Average K^+ currents at −80 mV. Data are averaged from four to nine oocytes for each condition.

CIR mRNA is present in several brain regions (39) but is notably poorly expressed in cerebral cortex and in the cerebellum (39), two brain regions in which GIRK1 is abundantly expressed (24). These results imply that these two inwardly rectifying K^+ channels are not always associated and suggest heteromultimerization of GIRK2 or GIRK3 with GIRK1 in some brain areas. Moreover, it is interesting to note that in dorsal raphe and locus coeruleus neurons, despite the low levels of GIRK1 mRNA (24), a robust G-protein-activated conductance is described (14–17). Expression of still undescribed GIRKs in these brain regions may explain these observations.

GIRK3 expression did not result in expression of measurable functional channels even by coexpression of G protein subunits $G\beta_1$ and $G\gamma_2$. The facts that GIRK3 does enhance the G-protein-evoked responses with GIRK1 coexpression and suppresses the G-protein-evoked responses with GIRK2 coexpression indicate that GIRK3 can coassemble with GIRK1 and GIRK2. The reason why GIRK3 alone does not give functional channels in oocytes is not known. It may not be targeted to the plasma membrane without a partner; alternatively, it may not bind activated G proteins.

A very important issue raised by the occurrence of heteromultimeric channels is their differential distribution in excitable tissues and the physiological significance. Distinct GIRK heteromultimers may be modulated or activated by G proteins differentially, as reported for other G protein effectors, such as for the several isoforms of adenylate cyclase (40); this would allow a larger degree of flexibility on the control of this important form of neuronal signaling.

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