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A Novel Ubiquitously Distributed Isoform of GIRK2 (GIRK2B) Enhances GIRK1 Expression of the G-Protein-Gated K+ Current in *Xenopus* Oocytes

Shojiro Isomoto, Chikako Kondo, Naohiko Takahashi, Shigeto Matsumoto, Mitsuhiko Yamada, Toru Takumi, Yoshiyuki Horio, and Yoshihisa Kurachi[1]

Department of Pharmacology II, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565, Japan

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We have isolated a novel variant form of GIRK2, designated GIRK2B, from mouse brain cDNA library. GIRK2B was much shorter than the first type of GIRK2 (GIRK2A), but its amino acid sequence was identical to the corresponding part of GIRK2A except the C-terminal eight amino acid residues. When GIRK2B cRNA was co-injected with GIRK1 and m_2-receptor cRNAs to *Xenopus* oocytes, acetylcholine-induction of the inwardly rectifying K^+ current was enhanced dramatically. This suggests that GIRK2B can form a heteromultimeric G-protein-gated K^+ channel with GIRK1. The reverse transcription polymerase chain reaction analysis showed that GIRK2B mRNA distributed much more broadly than GIRK1 mRNA. Therefore, GIRK2B might also play other unrecognized roles in various tissues than to form a K^+ channel with GIRK1.

The G-protein-gated inwardly rectifying K^+ (K_G)[2] channels exist in the brain as well as in the heart. The clone (GIRK1/KGA), which encodes the main subunit of K_G channel, was isolated from the rat heart [1,2]. From a mouse brain cDNA library, two additional clones homologous to GIRK1 were further obtained and designated GIRK2 and GIRK3 [3]. The rcK_{ATP}-1, which was initially reported to encode the ATP-sensitive K^+ channel, has ~70% homology in the amino acid sequence to GIRK1, thus may also belong to the family of GIRK [4]. Actually, it was shown that the cardiac K_G channel is composed of GIRK1 and CIR (cardiac inward rectifier), which is almost identical to rcK_{ATP}-1 [5]. It was also reported that, when either GIRK2 or GIRK3 was co-expressed with GIRK1, the G-protein-activation of K^+ current was prominently enhanced [6,7]. These results suggest that members of GIRK family can associate with each other to form functional heteromultimeric K_G channels. Therefore, it is important to identify the clones which potentially are subunits of K_G channels for elucidating the molecular mechanism underlying G-protein-activation of K_G channels in various tissues.

In the present study, we have tried to find a new member of GIRK family. We have isolated a novel variant form of GIRK2 from mouse brain cDNA library and designated this clone as GIRK2B. We examined the electrophysiological properties of GIRK2B using the *Xenopus* oocyte expression system and also tissue distribution of its mRNA by reverse transcription polymerase chain reaction (RT-PCR) technique.

MATERIALS AND METHODS

Screening of mouse brain cDNA library and DNA sequencing. A mouse brain cDNA library (Stratagene, La Jolla, CA) was screened under a mild stringency condition using *Pst* I digested human cK_{ATP}-1 cDNA (~1.5 kb) as a probe. Human cK_{ATP}-1 cDNA was kindly provided by Dr. J. P. Adelman (Oregon Health Sciences University, Portland, OR) [4].

[1] Correspondence should be addressed to Y. Kurachi at Department of Pharmacology II, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565, Japan. Fax: +81-6-879-3519.

[2] The abbreviations used are: K_G channel, G-protein-gated inwardly rectifying K+ channel; C-terminus, carboxy-terminus; N-termini, amino-termini; RT-PCR, reverse transcription polymerase chain reaction; $G_{\beta\gamma}$, $\beta\gamma$ subunits of G protein; ACh, acetylcholine; S.E.; standard error; bp, base pairs.

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Hybridization was conducted in 30% formamide, 5 × SSC, 5 × Denhaldt's solution, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, at 37°C for 18 h. Filters were washed with 2 × SSC, 0.1% SDS at room temperature for 20 min and then exposed to x-ray film overnight at −80°C with an intensifying screen. DNA sequencing was performed on both strands using a DNA sequencer (DSQ-1000, Shimadzu, Kyoto, Japan) as described previously [8].

Functional expression in Xenopus oocytes and electrophysiological measurements. The methods of preparation of oocytes, cRNA injection and electrophysiological measurements have been previously described [8]. *Xenopus* oocytes were injected with m_2-muscarinic receptor cRNA (10 ng per oocyte), as well as cRNAs for mouse brain GIRK1 (20 ng), GIRK2B (20 ng), or both GIRK1 and GIRK2B (10 ng each). After injection, oocytes were incubated in a modified Barths' solution at 18°C, and electrophysiological studies were undertaken 3 days later. Two-electrode voltage clamp experiments were carried out with a voltage clamp amplifier (Turbo Clamp TEC 01C, Tamm, Germany). Oocytes were bathed in a solution which contained 90 mM KCl, 3 mM $MgCl_2$, 5 mM HEPES, pH 7.4, and 300 μM niflumic acid to block endogenous chloride current. Voltage steps (1.2 sec in duration) to various potentials between +60 and −120 mV from the holding potential of 0 mV were applied every 7 sec. Results were expressed as mean ± S.E. Significant difference between values was assessed by Student's unpaired *t*-test or by Chi-square test. The p value <0.05 was considered significant.

RT-PCR amplification. Total RNAs from various organs were extracted by guanidine thiocyanate methods [9]. Complementary DNAs synthesized from these total RNAs with oligo-(dT) primers were used as templates for PCR amplification. The sequences of the primers were as follows: GIRK1, 5′-CTTCCATCGAAGCTGCAG-3′ (forward), and 5′-GTTTTGCTATGTGAAGCG-3′ (reverse), the sequences corresponding to nucleotides 1195-1212 and 1495-1512; GIRK2A, 5′-AATGACGTGCCAAGCCCGAA-3′ (forward), and 5′-ACACTAGGAGCCCAGCATCA-3′ (reverse), the sequences corresponding to nucleotides 954–973 and 1243–1262; GIRK2B, 5′-CTGTGAGAAATGCATTCA-3′ (forward), and 5′-GGCTTGATAACAAATAGC-3′ (reverse), the sequences corresponding to nucleotides 979-996 and 1471-1488. The PCR condition was as follows: an initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturing at 94°C for 45 sec, annealing at 58°C for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 8 min. Amplified DNA fragments were electrophoretically fractionated on 2% agarose gels.

RESULTS

The screening of mouse brain cDNA library with the human cK_{ATP}-1 as a probe [4] resulted in isolation of a 1.7 kb clone encoding 327 amino acids with two hydrophobic putative membrane-spanning domains (M1 and M2) and one potential pore-forming domain (H5) (Fig. 1A). The amino acid sequence of the resulting clone was identical from position 1 to 318 to that of GIRK2 reported by Lesage et al. [3], except for two amino acid residues at positions 260 and 313. The nucleotide sequence of the 5′-untranslated region of this clone was also identical to that of GIRK2. In contrast, the carboxy (C)-terminus of this clone was much shorter than that of GIRK2. The C-terminal nucleotide sequences including 3′-untranslated regions of this clone and GIRK2 were divergent from position 954. Thus, eight amino acid residues in the C-terminus of this clone were different from those of GIRK2 (Fig. 1B). Based on these results, we considered this clone to be an isoform of GIRK2 and designated this new clone as GIRK2B and the first type of GIRK2 as GIRK2A. The two divergent residues in the highly homologous parts of GIRK2A and GIRK2B were Ser^{260} (AGT) and He^{313} (ATC), and Thr^{260} (ACT) and Met^{313} (ATG), respectively. However, when we reanalized the sequence of GIRK2A which we obtained by utilizing RT-PCR technique from mouse brain mRNAs, these residues were Thr^{260} (ACT) and Met^{313} (ATG). Thus, the amino (N)-termini of GIRK2B and GIRK2A may be identical.

In Figure 2, representative current records from *Xenopus* oocytes injected with either GIRK1 cRNA alone (A), GIRK2B cRNA alone (B), or both (C) are shown. The cRNA for m_2-muscarinic receptor was always co-injected into oocytes. Currents measured in the control bathing solution and under application of 10 μM ACh in the three cases (A-C) are depicted in the first and second columns, respectively. The third column shows the ACh-induced currents which were obtained by subtracting the control currents from those under application of ACh at each potential. The ACh-induced currents were antagonized by 10 μM atropine. ACh did not induce any significant current alterations in the oocytes where only m_2-receptor were expressed (data not shown). The steady-state current-voltage relationships of the ACh-induced currents in the three different combinations of GIRK1 and GIRK2B are shown in Figure 2D. All of the ACh-induced currents rectified inwardly at potentials more positive than 0 mV. The frequency of successful expression in the

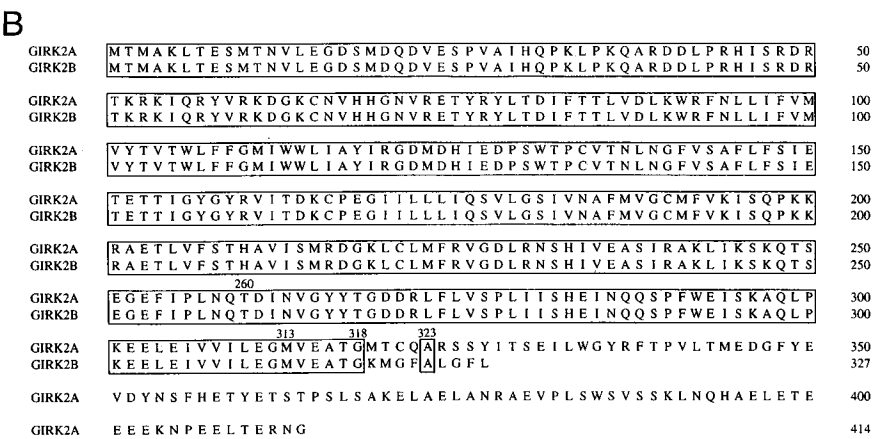

FIG. 1. The nucleotide sequence and deduced amino acid sequence of the GIRK2B cDNA (A) and alignment of the amino acid sequences of GIRK2A and GIRK2B (B). Nucleotide residues are numbered from the first nucleotide of the initiating ATG codon. Amino acids are numbered beginning with the initiating Met. The proposed trans-membrane regions are underlined. The potential pore-forming region is double underlined. GIRK2B is identical to GIRK2A in the N-terminal sequence corresponding to amino acid residues of 1-318 and divergent in the C-terminal sequence from amino acid residues of 319. The identical amino acid residues are boxed.

combination of GIRK1 and GIRK2B was 79% (n = 19), which was significantly greater than that of GIRK1 alone (14%, p = 0.003; n = 7) and that of GIRK2B alone (29%, p = 0.004; n = 14). As shown in Figure 2E, the steady-state current induced by ACh at −120 mV with the combination of GIRK1 and GIRK2B (0.22 ± 0.03 μA) was ~20 fold greater than that with GIRK1 alone (0.01 ± 0.01 μA, p = 0.0003) and ~10 fold greater than that with GIRK2B alone (0.02 ± 0.01 μA, p = 0.0001).

To compare the expression of GIRK1, GIRK2A and GIRK2B mRNAs, the RT-PCR assay was performed (Fig. 3). The specific PCR primers for amplification of GIRK1, GIRK2A, and GIRK2B were designed from the 3′ region of each cDNA sequence to produce cDNA fragments of 318 base pairs (bp), 309 bp and 510 bp, respectively. These regions were divergent among these three GIRK cDNA sequences. The RT-PCR yielded visible amplified product of GIRK1 in mRNAs of fore-brain, cerebellum, eye, atrium and skeletal muscle, and that of GIRK2A in forebrain, cerebellum and eye. On the other hand, the RT-PCR product of GIRK2B with expected length of the fragment

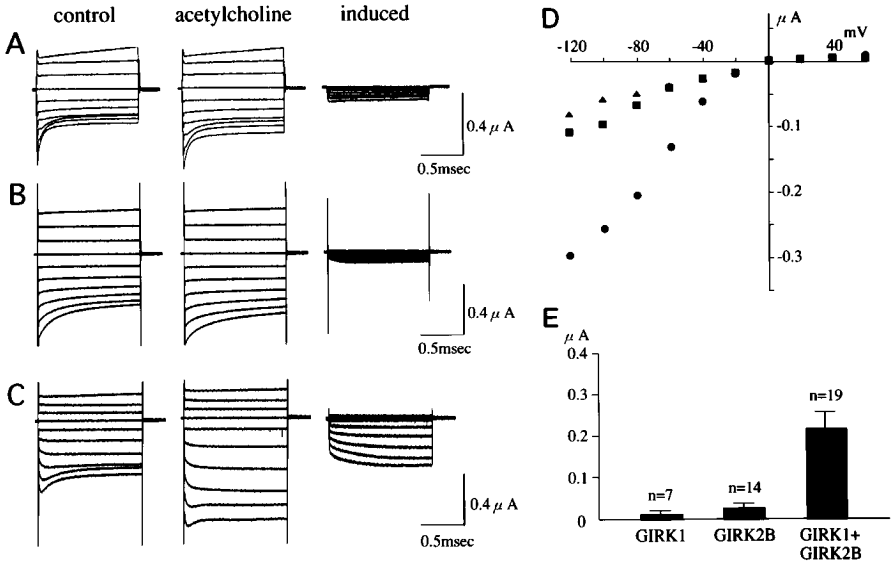

FIG. 2. Currents recorded from representative *Xenopus oocytes* expressing either GIRK1 or GIRK2B cRNA or both by two-electrode voltage clamp. Injection of either GIRK1 (A) or GIRK2B cRNA (B) or both (C) produced the inwardly rectifying currents. The cRNA for m_2-muscarinic receptor was always injected in *Xenopus* oocytes: Currents were measured during perfusion with the control bathing solution (first column) and during perfusion with the solution containing 10 μM ACh (second column). The third column presents the subtracted ACh-induced currents. Currents were measured 3 days postinjection. (D) The steady-state current-voltage relationships of the ACh-induced currents for representative *Xenopus* oocytes expressing GIRK1 (▲), GIRK2B (■) or both (●): The ACh-induced currents inwardly rectified in any case. (E) Average steady-state currents induced by ACh at −120 mV: The ACh-induced current with both GIRK1 and GIRK2B (0.22 ± 0.03 μA; n = 19) was significantly greater than that of GIRK1 alone (0.01 ± 0.01 μA, p = 0.0003; n = 7) and that of GIRK2B alone (0.02 ± 0.01 μA, p = 0.0001; n = 14).

(510 bp) was detected ubiquitously in forebrain, cerebellum, eye, atrium, ventricle, lung, stomach, colon, liver, pancreas, spleen, kidney and skeletal muscle.

DISCUSSION

In this study, we showed that co-expression of a novel variant form of GIRK2 (GIRK2B) with GIRK1 and m_2-muscarinic receptor enhanced the ACh-induction of inwardly rectifying K^+ currents in *Xenopus* oocytes. In the oocytes co-injected with GIRK1, GIRK2B and m_2-receptor cRNAs, the frequency of successful expression became 3–5 times greater and the amplitude of ACh-induced K^+ currents became 10–20 times larger, when compared with the currents in the oocytes injected with either GIRK1 or GIRK2B cRNA and m_2-receptor cRNA. This cannot be explained by a simple additive effect of co-expression of GIRK1 and GIRK2B. Therefore, it is suggested that GIRK2B can form a heteromultimeric K_G channel with GIRK1, as reported in the cases of CIR (GIRK4), GIRK2A and GIRK3 [5–7].

GIRK2B is considered to be an isoform of GIRK2A with divergent C-terminal end. Another isoform of GIRK2, different from GIRK2A or GIRK2B, which has a C-terminus II amino acids longer than that of GIRK2A, has been pointed out [3]. These findings may indicate that at least three different isoforms of GIRK2 can be generated by alternative splicing of transcripts from a single gene. In the inwardly rectifying K^+ channels, alternatively spliced isoforms of ROMK1 were also identified [10]. ROMK channel isoforms (ROMK1-3) expressed differentially in the kidney, but the electrophysiological properties of the expressed K^+ currents remained the same. Because GIRK2B enhanced GIRK1-expression of the G-protein-gated K^+ current in *Xenopus* oocytes as GIRK2A does, a major difference between GIRK2A and GIRK2B may also be their tissue dis-

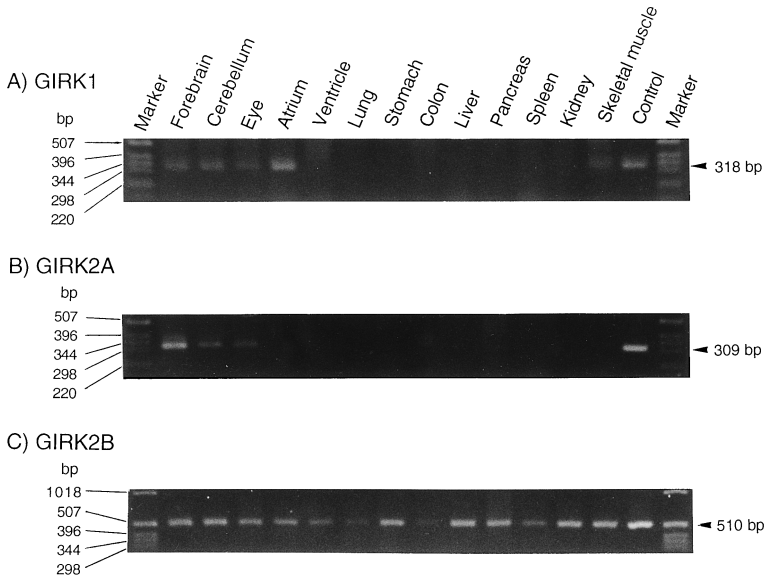

FIG. 3. RT-PCR detection of GIRK1, GIRK2A and GIRK2B mRNAs in various tissues. The RT-PCR assay was performed as described under Materials and Methods. Amplified fragments were electrophoretically fractionated on 2% agarose gels. Molecular weights were indicated by using the size marker shown at both ends. The RT-PCR resulted in visible amplified products with forebrain, cerebellum, eye, atrium and skeletal muscle mRNAs of GIRK1 (A), with forebrain, cerebellum and eye mRNAs of GIRK2A (B), and with forebrain, cerebellum, eye, atrium, ventricle, lung, stomach, colon, liver, pancreas, spleen, kidney and skeletal muscle mRNAs of GIRK2B (C).

tribution; i.e., GIRK2A mRNA was specific to the brain, while GIRK2B mRNA was ubiquitously distributed. Therefore, the transcriptional expression of GIRK2 mRNAs should be regulated in a tissue-specific manner.

It has been shown that members of the GIRK family (e.g. GIRK1, GIRK2A, GIRK2B, GIRK3, and GIRK4) can associate with each other when co-expressed in *Xenopus* oocytes [5–7]. Because mRNAs of all members of this family so far isolated are expressed in the brain [1–4,11,12], it is possible that the K_G channels are divergent and composed of heterologous combinations of members of the GIRK family including GIRK2B in the brain. To prove this hypothesis, the immunoprecipitation experiment is necessary. The same possibility can be applied to other organs than brain. Although the K_G channel activity coupled to a variety of receptors, such as m_2-muscarinic and opioid receptors [13,14], has been electrophysiologically identified in heart and brain, there is no report on receptor-dependent activation of K_G channels in other organs where the GIRK mRNAs are expressed: e.g., skeletal muscle contains the GIRK1 and GIRK2B mRNAs. Similarly, both GIRK2B and GIRK4 mRNAs are ubiquitously distributed, including lung, spleen, liver and kidney [4]. These results suggest a possibility that GIRKs may play other unrecognized functional roles in various tissues than to merely form K_G channels.

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