RGS7 and RGS8 Differentially Accelerate G Protein-mediated Modulation of K⁺ Currents*

(Received for publication, December 11, 1998, and in revised form, January 28, 1999)

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The recently discovered family of RGS (regulators of G protein signaling) proteins acts as GTPase activating proteins which bind to α subunits of heterotrimeric G proteins. We previously showed that a brain-specific RGS, RGS8 speeds up the activation and deactivation kinetics of the G protein-coupled inward rectifier K⁺ channel (GIRK) upon receptor stimulation (Saitoh, O., Kubo, Y., Miyatani, Y., Asano, T., and Nakata, H. (1997) Nature 390, 525–529). Here we report the isolation of a full-length rat cDNA of another brain-specific RGS, RGS7. In situ hybridization study revealed that RGS7 mRNA is predominantly expressed in Golgi cells within granule cell layer of cerebellar cortex. We observed that RGS7 recombinant protein binds preferentially to Goα, Goβ, and Goγ. When co-expressed with GIRK1/2 in Xenopus oocytes, RGS7 and RGS8 differentially accelerate G protein-mediated modulation of GIRK. RGS7 clearly accelerated activation of GIRK current similarly with RGS8 but the acceleration effect of deactivation was significantly weaker than that of RGS8. These acceleration properties of RGS proteins may play important roles in the rapid regulation of neuronal excitability and the cellular responses to short-lived stimulations.

Numerous extracellular signals such as hormones, neurotransmitters, and odors stimulate seven transmembrane-spanning receptors that activate heterotrimeric G proteins. These G proteins function as signal transducing molecules by regulating cellular effectors including enzymes and ion channels (1, 2). The regulatory mechanisms that control G protein signaling have not been fully studied. Recently, a new family of regulators of G protein signaling (RGS) was identified in organisms (3). Members (RGS1, RGS4, GAIP, RGSr/RGS16, RET-GAL) attenuate G protein signaling in cultures (9, 16, 17). Biochemical studies have demonstrated that some RGS members (RGS1, RGS4, RGS10, GAIP, RGSr/RGS16, RET-GAL) function as GTPase-activating proteins (GAPs) for the Gi family of α subunit, including Goαα, Goαβ, and transducin (10, 14, 18–20). Hence, these characterized RGS proteins are proposed to down-regulate G protein signaling in vivo by enhancing the rate of Gα GTP hydrolysis. However, whether other RGS proteins regulate G protein signalings in a similar manner remains to be established.

Because there are many G protein signaling pathways which regulate important functions such as neural transmission in the brain, it is possible that certain RGS proteins might determine a mode of G protein signaling that control neural functions. We searched RGS proteins specifically expressed in neural cells using neurally differentiating P19 cells in culture. RGS8 was induced in neurally differentiated P19 cells. Biochemical studies indicated that RGS8 functions as a GAP for Goα and Goαγ. To examine effects of RGS8 on G protein signaling, we co-expressed a G protein-coupled receptor and a G protein-coupled inwardly rectifying K⁺ channel (GIRK1/2) (21–23) in Xenopus oocytes and analyzed turning on and off upon agonist application. We found that RGS8 significantly accelerates both turning on and off (24). Do other neural-tissue-specific RGS proteins function in a similar manner? RGS4 and RGS7 have been reported to be expressed predominantly in the brain (8, 9). Doupnik et al. showed similar electrophysiological results on RGS1, 3, and 4 as RGS11 (25). As only partial sequence with incomplete 5' end of RGS7 cDNA has been known (8), we isolated a cDNA clone encoding full-length RGS7 for functional analysis. We determined distribution of RGS7 mRNA and binding character of the core domain of RGS7 and analyzed effects of RGS7 on turning on and turning off kinetics of GIRK current.

**EXPERIMENTAL PROCEDURES**

Cloning of RGS7 cDNA—Oligonucleotide primers (5'-TTGGCGATGTGAGCACTCTGGAGAAAG-3', 5'-CAGCTTGAATGTGCTCCTTGGAGCAT-3') were synthesized based on the reported sequence of rat RGS7 (8). Total RNA was isolated from rat brain, and the first strand cDNA was synthesized as a template of PCR. The amplified 200-base pair DNA was cloned into pGEM-T vector (Promega), and its sequence was determined. This PCR-amplified fragment of RGS7 was used to screen rat hippocampus cDNA library. The longest cDNA clone was sequenced on both strands.

Northern Blot—Northern blot analysis was performed as described previously (26). Digoxigenin-labeled cRNAs, which had been synthesized using RGS7 and RGS8 cDNAs, were utilized as a probe. To detect...
mRNA of neurofilament protein 140K and glyceraldehyde-3-phosphate dehydrogenase, digoxigenin-labeled DNA probe was also prepared by random priming method. In Situ Hybridization—In situ hybridization was performed as described (27) with the following modifications. Sagittal frozen sections of rat brain were treated with 5 \( \mu \text{g/ml} \) proteinase K for 5 min at 37 °C. To remove excess cRNA probes after hybridization, RNase A treatment (20 \( \mu \text{g/ml}, 30 \text{ min}, 37 ^\circ \text{C} \)) was carried out. Expression and Purification of His-tagged RGS7 Core Domain—Recombinant protein of RGS core domain of RGS7 was expressed with hexahistidine tag at the N terminus in \textit{Escherichia coli}. Bacterial expression plasmid of His-tagged RGS7 was constructed as follows. The coding region corresponding to amino acids 303–470 of RGS7, which includes RGS core domain, was PCR-amplified using RGS7 cDNA as a template. Nucleotide sequence of the amplified DNA was confirmed by sequencing both strands. The confirmed DNA fragment was cloned into pQE30 (Qiagen). The resultant plasmid was transformed into \textit{E. coli}, M15. His-tagged RGS7 core domain, which was induced by IPTG treatment (5 mM, 1 h), was purified using Ni\(^{2+}\)-NTA-agarose as described previously (24). Binding Assay of RGS7—Binding assay between RGS protein and G protein was carried out as previously described (24). His-tagged RGS protein (10 \( \mu\text{g} \)) and rat brain membrane fractions (0.5 mg) were incubated for 30 min at 5 °C in 20 \( \mu\text{M} \) HEPES, pH 8.0, 0.38 M NaCl, 3 mM dithiothreitol, 6 mM MgCl\(_2\), 10 \( \mu\text{M} \) GDP, 30 \( \mu\text{M} \) AlF\(_4^\text{2-}\) (30 \( \mu\text{M} \) AlCl\(_3\), 10 mM NaF), 40 mM imidazole. After solubilization with 1% cholate for 1 h at 4 °C and centrifugation at 30,000 rpm for 20 min, Ni\(^{2+}\)-NTA-agarose beads were added to detergent-soluble extract, incubated for 30 min at 4 °C, and washed five times with 20 mM HEPES, pH 8.0, 3 mM dithiothreitol, 0.1% polyoxyethylene 10-lauryl ether (C\(_{12}\)E\(_{10}\)), 1 \( \mu\text{M} \) GDP, 30 \( \mu\text{M} \) AlF\(_4^\text{2-}\), and 40 mM imidazole. Complexes containing His-tagged RGS protein were eluted from Ni\(^{2+}\)-NTA-agarose with SDS sample buffer and examined by SDS-polyacrylamide gel electrophoresis. The identification of proteins bound to RGS protein was performed by immunoblotting.

**Fig. 1. Rat RGS7 Sequence.** Predicted amino acid sequence of RGS7 derived from the rat RGS7 cDNA sequence was aligned with that of mouse RGS7. Amino acids identical in both sequences are boxed. The amino acid sequences underlined correspond to RGS domain.

**Fig. 2. Northern blot analysis of expression of RGS7 and RGS8 mRNAs.** A, total RNA was isolated from various rat tissues: brain (1), heart (2), lung (3), stomach (4), spleen (5), liver (6), kidney (7), testis (8), and back muscle (M. Latissimus dorsi, 9). 20 \( \mu\text{g} \) of isolated RNA was electrophoresed, transferred, and then hybridized with full-length rat RGS7 cRNA, full-length rat RGS8 cRNA, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Arrowheads indicate the positions of 28S rRNA. B, total RNA (10 \( \mu\text{g} \)) was isolated from developing brains; heads of 13-day embryos (1), brains of 14-day, 15-day, 17-day, 19-day, and 21-day embryos (2–6), 6-day, 13-day neonates (7 and 8), and adults (9). Expression levels of RGS7, RGS8, and neurofilament protein 140K (NF140K) mRNAs were examined by Northern hybridization. Arrowheads indicate the positions of 28S rRNA. C, brain stem (1), cerebral cortex (2), and cerebellum (3) were dissected from adult brains, and total RNA (10 \( \mu\text{g} \)) was isolated. Expression levels of RGS7, RGS8, and RGS4 mRNAs were examined by Northern hybridization. Arrowheads indicate the positions of 28S rRNA.
blotting with antibodies to Ga\(_{i1}\), and Ga\(_{i2}\), Ga\(_{i3}\), (Calbiochem-Novabiochem), Ga\(_o\), Ga\(_z\), and Ga\(_q/11\) (Santa Cruz Biotechnology). Signals were detected with ECL system (Amersham Pharmacia Biotech).

Two Electrode Voltage Clamp—Two electrode voltage clamp analysis was carried out as described previously (24).

RESULTS AND DISCUSSION

Isolation of Rat RGS7 cDNA—The sequence of RGS domain of rat RGS7 was previously reported (8), and the corresponding DNA fragment was obtained by PCR amplification using rat brain cDNA as a template. This PCR-amplified fragment was utilized to screen rat hippocampus cDNA library. The nucleotide sequence of the isolated longest clone was determined. Rat RGS7 cDNA of 2243 base pairs encoded a protein of 477 amino acids. While performing this work, isolation of bovine and mouse RGS7 was reported (28). Comparison of amino acid sequence revealed that our rat RGS7 was 94.5% identical to mouse RGS7 (Fig. 1). The conserved region among RGS proteins, the RGS domain, was present near C-terminal tail (amino acids 330–447). As reported for bovine RGS7 (28), a domain of 190 amino acids near the N terminus showed a considerable sequence identity to bovine RGS9 and Caenorhabditis elegans EGL10 (8).

RGS7 Expression Is Developmentally and Regionally Regulated—Expression levels of rat RGS7 mRNA in various tissues of the brain were examined by Northern hybridization, and brain-specific expression of RGS7 was confirmed (Fig. 2A). Whole brains were isolated from developing rats, and expression patterns of RGS7 and RGS8 mRNAs were compared. RGS7 expression was detected in 17-day embryos, increased gradually in later embryos, and peaked in 13-day neonates. In the case of RGS8, expression was detectable even in 13-day embryos and increased to adults (Fig. 2B). Brain stem, cerebral cortex, and cerebellum were dissected, and levels of mRNA expression of three RGS proteins, which are predominant in brains (RGS4, RGS7, and RGS8) were compared. Both mRNAs of RGS7 and RGS8 were most abundantly expressed in cerebellum, although RGS4 mRNA was abundant in cerebral cortex.

We next examined the cellular distribution of transcripts of RGS7 and RGS8 by in situ hybridization using nonradioactive probes. RGS7 mRNA was expressed in cerebral cortex, especially in layers 2 and 3. RGS7 was also detected in hippocampus and cerebellum (data not shown, Fig. 3A). Similar distribution patterns of RGS7 were previously reported with \(^{35}\)S-labeled probes (29, 30). In addition, we observed that RGS7 mRNA was most strongly expressed in medium-sized cells in the granule cell layer of cerebellar cortex. These cells were larger in size than the granule cells and were identified as Golgi cells (Fig. 3A). The probe for RGS8 mRNA densely labeled Purkinje cells of cerebellum (Fig. 3B). Thus, it is clearly
demonstrated that RGS7 and RGS8 mRNAs are expressed in distinct inhibitory neurons in cerebellum.

**RGS7 Binds Ga<sub>a</sub>, Ga<sub>i3</sub>, and Ga<sub>z</sub>—**

By biochemical in vitro binding method, we examined the specificity of RGS7. Popov et al. demonstrated functional similarity between the full-length RGS4 protein and its RGS domain in regard to affinities toward activated Ga<sub>a</sub>, catalytic activities, and acceleration values. Moreover, they also showed that specificity of the RGS domains of RGS4, GAIP, and RGS10 for Ga<sub>a</sub> subunits are similar to the reported specificity of full-length RGS proteins (31). Thus, RGS domain is generally considered to be sufficient to determine the basic properties of RGS proteins. We prepared recombinant protein corresponding to amino acids 303–470, covering the RGS domain of RGS7, using E. coli as a hexahistidine-tagged protein. This RGS7 core domain (RGS domain) protein (RGS7d) was purified and incubated with brain membranes treated with GDP and AlF<sub>4</sub>. Complexes containing RGS7d were precipitated with Ni<sup>2+</sup>-NTA-agarose and were analyzed by SDS-polyacrylamide gel electrophoresis. After Coomassie Blue staining, we observed that a 40-kDa protein recovered with RGS7d (Fig. 4A). To determine which Ga<sub>a</sub> is recognized with RGS7, we performed immunoblotting using antisera specific for different Ga<sub>a</sub> subunits.
subtypes of Go subunit. When proteins recovered with RGS7d were examined, we found that GoGα and GoGα were also recovered with RGS7d in addition to GoGα (Fig. 4B). Thus, it was clarified that RGS7 recognizes GoGα, GoGα and GoGα. We previously demonstrated that RGS8 binds only GoGα and GoGα (24). GoGα is a member of Gi family of a subunit, and interaction with GoGα has been reported for GAIP, RGS4, and RGS10 (20, 31, 32). Here we observed that RGS7 also binds GoGα, indicating that RGS7 and RGS8 have different selectivity of GoGα bindings in brain membranes where various types of GoGα exist.

It was reported that RGS7 inhibited GoGα-coupled calcium mobilization (30). We, however, could not detect GoGα by immunoblotting of proteins precipitated with the RGS7d. It is possible that RGS7 interacts with GoGα weakly but that their binding affinity is not strong enough for in vitro co-precipitation. Because an apparent and intense band was visualized by immunoblotting of brain membranes with GoGα antibody (Fig. 4B, top panel), the possibility that the GoGα present in the used membranes was degraded was excluded.

RGS7 and RGS8 Differentially Accelerate G Protein-mediated Modulation of K+ Currents—GIRK are known to be activated directly by Gβγ subunits released from pertussis toxin-sensitive G proteins of Gi family including Gi and Go (33, 34). They are activated by various G protein-coupled receptors such as m2 muscarinic and D2 dopamine receptors. The expression patterns of GIRK family (GIRK1–GIRK4) mRNAs in the brain are known in detail. It was shown that the brain type GIRKs (GIRK1 and GIRK2) are widely distributed and expressed in hippocampus and cerebral cortex (35). Thus, expression of GIRK1 and GIRK2 partly overlaps with RGS7 expression. Coexpressed GIRK1/GIRK2 heteromultimer and m2 muscarinic receptor with or without RGS protein in Xenopus oocytes and analyzed the speed of turning on and off agonist application under two-electrode voltage clamp (Fig. 5A). As previously reported, coexpression of RGS8 obviously accelerated the speed of both turning on and off. RGS7 coexpression accelerated the turning on process similarly with RGS8. The effect of RGS7 on off-acceleration, however, was much weaker than that of RGS8. This observation was statistically confirmed by comparing the time constants for the fitted single exponential function of the activation and deactivation phases, τon and τoff (Fig. 5, B and C).

We previously reported that RGS8 has a function to increase the on rate besides the GAP activity and that the on-acceleration cannot be explained by the GAP activity itself. Chuang et al. (36) also showed that RGS4 has both a negative regulator function as a GAP and a positive regulator function to increase the available G protein pool. They discussed various possible mechanisms of the latter, including GAP activity itself. Our observation that on acceleration and off acceleration are not parallel modulated by RGS7 and RGS8 supports that on and off acceleration is regulated by distinct mechanisms. The biochemical basis of on acceleration remains to be elucidated.

Chuang et al. (36) concluded that the acute desensitization of the response is because of the nucleotide exchange and hydrolysis cycle of G proteins. We also observed that the desensitization speed is accelerated by RGS proteins similarly with the off acceleration, i.e. the desensitization with RGS8 was faster than with RGS7.

What is the structural basis for the weaker off acceleration effect of RGS7? It is most likely that lower GAP activity might contribute to weak acceleration of off process. However, recombinant protein of RGS7 core domain has been shown to act as a strong GAP for Go subfamily proteins in vitro (30), and its high activity does not seem to be significantly different from that of RGS8, which we previously reported (24). Another possibility is that domain(s) other than RGS domain, which for example determine the subcellular localization of RGS protein, could affect the function. A characteristic structural feature of RGS7 among RGS proteins is its long N terminus. Koelle and Horvitz (8) demonstrated that the protein of EGL10, which is a C. elegans homologue of RGS7 is present in processes of neurons and in dense body/sarcoplasmic reticulum-like structures within body wall muscle cells. They demonstrated that N-terminal region of EGL10 functions to localize the protein at least within muscle cells using transgenic nematodes. It is possible that this N-terminal domain of RGS7 may function as a regulatory element of off acceleration of G protein signaling by changing subcellular localization of RGS7.

The effect of RGS protein on the dose-response (peak current) relationships upon receptor stimulation was analyzed (Fig. 5D). As described previously, RGS8 has almost no effects on the dose-response relationships (24). RGS7 shifted the relationship curve to lower dose only slightly. Thus, RGS 8 and RGS7 differentially accelerate the time course of G protein-mediated modulation of GIRK without significantly influencing the dose-response relationship of the induced currents. These different abilities of RGS proteins to speed up G protein-signalings may be physiologically important. On acceleration is thought to be useful for cellular responses to short-lived signals such as neurotransmitter release in the brains. As Doupnik et al. (25) described, without RGS proteins, brief pulse application of agonist leads to only a small amplitude of the GIRK current. On the other hand, with RGS proteins, brief agonist stimulation can still cause full activation of GIRK by on acceleration. Thus, with either RGS8 or RGS7, GIRK can be fully activated even by short application of agonist. With RGS8, however, because of highly accelerated deactivation, the integrated K+ current is less than that with RGS7, which only weakly speeds up the deactivation. The strong effect of RGS7 on acceleration and the weak effect on off acceleration are thought to enhance the integrated response caused by short agonist application very efficiently.

Acknowledgments—We are grateful to Prof. M. Lazdunski for GIRK2 cDNA and to Dr. A. Connolly for m2 muscarinic receptor cDNA. We thank Dr. H. Nakata for helpful discussion and K. Nakata for preparing sections for in situ hybridization.

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Difference of GIRK Response Acceleration by RGS7 and RGS8

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doi: 10.1074/jbc.274.14.9899

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