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Forskolin-Induced Stimulation of RGS2 mRNA in C6 Astrocytoma Cells

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RGS is a negative regulator of G-protein signaling and can be identified by the presence of a conserved 120~125 amino acid motif, which is referred to as the RGS box. A number of RGSs are induced in response to a wide variety of stimuli. Increased levels of RGSs lead to significant decreases in GPCR responsiveness. To obtain further evidence of a role of RGS proteins in rat C6 astrocytoma cells, we first determined the expression profile of RGS-specific mRNA in C6 cells using reverse transcription - polymerase chain reaction (RT-PCR) with a poly dT18 primer and transcript-specific primers. We found that RGS2, RGS3, RGS6, RGS9, RGS10, RGS12, and RGS16 were differentially expressed in C6 astrocytoma cells. The highest expression rate was found for RGS3, followed by RGS16, RGS10 and RGS9, whereas the expression level for RGS2 was barely detectable. We next assessed whether forskolin regulated the expression of RGSs expressed in C6 astrocytoma cells. The present study found that forskolin dose-dependently stimulated the expression of RGS2 transcripts. This up-regulation of RGS2 gene was abrogated by H-89, potent and broad-spectrum protein kinase A (PKA) inhibitors. Actinomycin D completely inhibited the up-regulation of RGS2 gene induced by forskolin (10 μM), indicating that the regulation of RGS2 gene is controlled at the transcriptional level. In addition, forskolin did significantly activate transcriptional cAMP response element (CRE) in either HEK 293 cells or C6 cells and did not modulate the NF-κB and AP-1 activity as measured by luciferase reporter gene assay. Finally, forskolin induced the expression of RGS2 mRNA in C6 astrocytoma cells, which depend on the PKA pathway and CRE transcriptional pathways.

Key Words: RGS; Astrocytoma cells; Forskolin; RT-PCR; PKA; CRE

INTRODUCTION

The G protein-coupled receptor (GPCR) system is ubiquitously found throughout the human body and is involved in virtually every pathophysiological process in human beings (Ja and Roberts, 2005; Pierce et al., 2002). It is composed of seven transmembrane-spanning receptors, heterotrimeric G proteins, and effectors (e.g. including mitogen-activated protein kinase [MAPK], phospholipase C [PLC], and phospholipase A_2 [PLA_2]) (Berman and Gilman, 1998; Ross and Wilkie, 2000). RGS proteins have recently been identified in mammals as the negative regulators of G protein signalling pathways, which have become the fourth component of the GPCR system (i.e. receptors, G proteins, effectors, and RGS) (Heximer et al., 1999; Watson et al., 1996). Effort to identify new RGS proteins has extended this family to approximately 25 members. All RGS proteins have the potential of attenuating G protein signalling by accelerating the rate of GTP hydrolysis through G protein α subunits called GTPase-accelerating proteins (GAP) (Berman and Gilman, 1998; De Vries et al., 2000; Dohlman and Thorner 1997; Ross and Wilkie, 2000). However, RGS proteins are not simply GAP (Dohlman and Thorner, 1997). Certain

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RGS proteins can function as effector antagonists in G protein signaling pathways, but others can also serve effectively with effectors (De Vries et al., 2000). Since inhibition of GPCR signaling by RGS occurs without affecting the receptor per se, RGSs are potential candidates to be involved in mediating decreases in GPCR responsiveness that are not due to the internalization of cell surface receptors. In addition, the level of G protein does not vary significantly under various physiological conditions, but several RGS mRNAs have been shown do so (Grant et al., 2000). This is strongly supported by the fact that a number of RGSs are induced in response to a wide variety of stimuli and that increased levels of RGSs lead to significant decreases in GPCR responsiveness. Thus, studying the expression and regulation of RGS proteins may elucidate the pathophysiology of GPCR-related disorders (Gertsch et al., 2004; Wang et al., 2002).

On the other hand, astrocytes are cell constituents of the CNS and occupy 20~30% of the brain volume. Astrocytes, named for their stellate apperarance, project foot processes that envelop the basement membrane of neurons, synapses, and capillaries, thereby consisting of the blood-brain barrier (BBB) (Porter and McCarthy, 1996). Astrocytes play an important role in metabolism of neurotransmitters and in mechanisms of neurotransmitters uptake that protect neurons from injury during glutamate toxicity (Rothstein et al., 1996). Astrocytes also express increased levels of neurotrophic factors in response to pathological conditions in the CNS such as Alzheimer's Disease (AD). Indeed, astrocytes act as mediators of inflammatory responses and are implicated in the pathophysiology of neurodegenerative diseases including Alzheimer's Disease (AD) and multiple sclerosis (MS) (Dorf et al., 2000). Cultured astrocytes express a wide variety of G protein - coupled receptors including β2-adrenoceptor, α1-adrenoceptor, and chemokine receptors (De Keyser et al., 2004; Kimelberg, 1995). Reactive astrocytes lead to an increase in the levels of intracellular cyclic adenosine-5-monophosphate (cAMP) (Kimelberg, 1995). Significantly astrocytes in MS patients lack the β2-adrenoceptor by which the mechanism is not known. Moreover, the activation of β2-adrenoceptor by agonists leads to morphological differentiation of primary astrocytes (Gharami and Das, 2004), to increased production of nerve growth factor of astrocytoma cells (Mocchetti et al., 1989), and to proliferation of reactive astrocytes sensitive to cytotoxicity of 7β-hydroxysterol (Bochelen et al., 2000). These phenomena seem to be attributable to the increased level of intracellular cAMP which thereby activates the astrocytes. Therefore, we investigated the relationship between cAMP's upregulation and induction of any RGS gene in astrocytoma cells. We examined the expression profiles of RGS mRNA, to determine whether forskolin, a direct activator of all known subtypes of adenylyl cyclase (AC) to produce cAMP, regulates the expression of RGS mRNA in C6 astrocytoma cells.

MATERIALS & METHODS

1. Materials

Firefly pNF-κB Luc and pCRE-Luc constructs were obtained from Stratagene Co (La Jolla, CA). A phRL-TK construct and a dual-Luciferase reporter assay system were from Promega Co (Madison, WI). Forskolin, Actinomycin D, and IBMX were obtained from Sigma (St. Louis, MO). Staurosporine, H-89, PD98059, SB203590, and SP600125 were from Tocris (Bristol, UK). A transfection kit was from Wellgene Co (Daegu, Korea).

2. Cell culture & transfection

Rat C6 cells and HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 5% FBS. Cells were grown at 37℃ and 5% CO_2 in humidified air and were passaged twice a week. Transient transfection of C6 cells was performed using Wellfect transfection kit (Wellgene Co., Daegu, Korea) according to the manufacturer's instructions. HEK 293 cells were transiently transfected with the calcium phosphate method.

3. Extraction of total RNA

Total RNAs from culture cells were prepared by adding easy-BLUE total RNA extraction reagent (iNtRON Co, Seoul), according to manufacturer's instructions. Total RNA was solubilized with 0.1% diethylpyrocarbonate (DEPC)-treated DDW and was stored at -70℃ until used.

4. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) amplification

Semiquantitative RT reactions were carried out using RT premix (Bioneer Co, Daejon). Total RNAs (2 μg) were incubated with oligo-dT_{18} at 70℃ for 5 min and cooled on ice for 3 min. Total RNA was then mixed with RT premix

for further incubateion at 37℃ for 60 min. The reactions were terminated at 95℃ after 10 minutes and a PCR reaction was further conducted with PCR premix (Bioneer Co, Daejon) under the following incubation conditions: a 45 second denaturation time at 94℃, an annealing time of 45 seconds at 55 to 60℃, an extension time of 60 seconds at 72℃, and a final extension of 7 minutes at 72℃ at the end of 35 cycles. Primers used in this experiment were obtained from Bioneer Co. (Table 1).

5. Luciferase assay

Either HEK 293 cells or C6 cells was seeded in 24 - well plates (1×10^6/ml) and maintained for 24 hr. Cells were transfected with pCRE-pAP-1, pSRE and pNFκB-luciferase reporter vector using appropriate reagents. Cells were then treated with test agents at the indicated concentrations for various times, and luciferase activity was measured using a luciferase assay kit (Promega, USA) and a luminometer (Turner BioSystems, USA).

6. Statistical analysis

The One-way ANOVA were used to determine the statistical significance of differences between values for the experimental and control groups. *P* values of 0.05 or less

Table 1. The sequences of primers and fragment sizes of the investigated genes

Gene		Primer sequences	Fragment size (bp)
RGS1	F	5'-CCA GAT GGG TCA AAA TGT CTT TGG-3'	228
	R	5'-GAG TTG GTG TTT TGA TCT TCT TGG-3'	
RGS2	F	5'-CAA AAC TCC TCT ACT CCT GGG AA-3'	372
	R	5'-GTT GTT CTC CAT CAG GCT GTA CA-3'	
RGS3	F	5'-CTT GTG AGG ATT TCA AGA AGG TC-3'	192
	R	5'-GAA GAT ACG TTT TTG TGC CAG GTC-3'	
RGS4	F	5`-CTT CTG GAT CAG CTG TGA GGA GTA C-3'	279
	R	5`-GCA GCT GGA AGG ATT GGT CAG GTC-3'	
RGS5	F	5'-CCA GAA GCC AGA CTC TGC TGT TGA C-3'	271
	R	5'-CAT AGA TTT GCT TTG CCT TCT CTG-3'	
RGS6	F	5'-TGG CCG TCC AAG ATC TCA AGA AGC-3'	191
	R	5'-GTA GAT GTG CTC CTG GGC ATC TTC-3'	
RGS7	F	5'-TCC AAC AGA AGA TGA GTT GCA CCG-3'	291
	R	5'-CTT AAG GAA CTG CTC TCT CCC AAC-3'	
RGS8	F	5'-CCA CGC AGG AAC AAA GGC ATG AGG-3'	245
	R	5'-CAT GAG GCT GTG GAC TTT TCC CTG-3'	
RGS9	F	5'-GAA GCA CTC GTG AAG GAC ATG CAG-3'	478
	R	5'-GCG CAT ACC TGT CAG CTT TGT TCC-3'	
RGS10	F	5'-GCG TGT GAA GAT TTG AAG AAA ACG-3'	191
	R	5'-GAG ATT GAA GAT CTG GTC CTG GAG-3'	
RGS11	F	5'-CGT GTG AGG AGC TGC GCT TTG GC-3'	186
	R	5'-GTA GAT GTG CAG TTG TGC TGC ATC-3'	
RGS12	F	5'-GGT GCT TGG CAC AGG AAG ATG AAG-3'	262
	R	5'-GCA TCC CCA TCT AGA AAG GCA CG-3'	
RGS13	F	5'-TG GAA ACA TTG CTT TTC TTT TCT C-3'	328
	R	5'-T AAC CAA ATG GCA ACA TCT GAT T-3'	
RGS14	F	5'-GAA GTA CTG CTG CTG GTA TCT ACC-3'	277
	R	5'-GGC TTA GCA GAG ATC CGT ACC AC-3'	
RGS16	F	5'-CAC CAT CTT CTT CGA TGT CAA AGG-3'	277
	R	5'-CAT GCT CCA GTC AAG CAA GAT GTC-3'	
RGS18	F	5'-C TTC CAT GGA GAG ACT CAA GCC AG-3'	316
	R	5'-AGA GTG GGC TGG GCG ATG CTC TTA GC-3'	
GAPDH	F	5'-CAC GGC TGC CTG CTG GCT CTG TAC C-3'	464
	R	5'-CCT TGG CAG CAC CAG TGG ATG CAG G -3'	

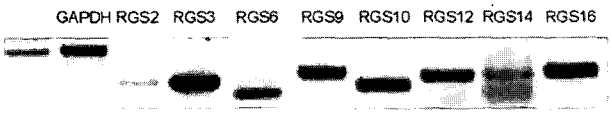

Fig. 1. Expression profiles of various RGS mRNA in C6 cells. The results of the gel electrophoresic separation of primer-specific PCR products.

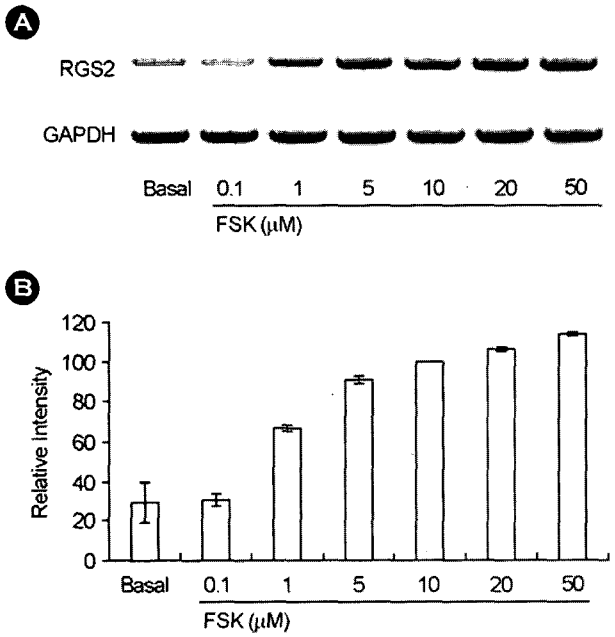

Fig. 2. Effect of forskolin with various concentrations on the expression of RGS2 mRNA in C6 cells. Qualitative **(A)** and quantitative results **(B)** of the gel electrophoresic separation of primer-specific PCR products. The columns indicate the expression rate relative to the expression of GAPDH given as mean ± SEM, n = 3.

were considered as statistically significant.

RESULTS AND DISCUSSIONS

1. The expression profiles of RGS2 mRNA in rat astrocytoma C6 cells

We first determined the mRNA expression pattern for the known RGS members in C6 cells. Due to the close similarities in the DNA sequences of the various RGS proteins, PCR reactions were performed using isotype-specific primers under conditions that resulted in a specific band for each RGS transcript. The gene-specific primers are listed in Table 1. The transcripts for the following RGS were found to be expressed in rat C6 cells: RGS2, RGS3, RGS6, RGS9, RGS10, RGS12, and RGS16 (Fig. 1). The expression level of RGS3, which is known to be specific regulator of $G_{\alpha q}$ subtype signalling, was most dominant in rat C6 cells as

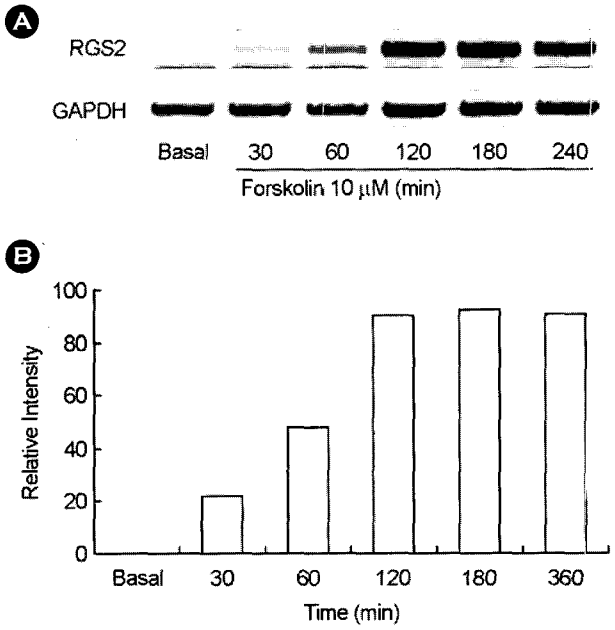

Fig. 3. Effect of forskolin at 10 μM with various incubation time on the expression of RGS2 mRNA by the various incubation time in C6 cells. Qualitative **(A)** and quantitative results **(B)** of the gel electrophoresic separation of primer-specific PCR products. The columns indicate the expression rate relative to the expression of GAPDH. The figure presents the representative results out of three experiments, which give similar results.

compared to other RGS proteins. The transcript of RGS2 mRNA was detectable in RT-PCR using poly dT_{18} primer and gene-specific PCR primers.

2. The effect of forskolin on the expression of RGS2 mRNA in rat astrocytoma C6 cells

It has been reported that the expression of RGS2 is regulated by a various signaling components of G protein and other factors, such as stress, cyclic adenosine-5-monophosphate (cAMP), and protein kinase C (PKC). We examined whether forskolin, a stimulator of all AC subtypes, regulates the expression of RGS mRNA transcripts in C6 cells using RT-PCR with gene-specific primers. Forskolin produced elevated RGS2 mRNA levels in a dose- and time-dependent manner (Fig. 2, 3). As shown in Fig. 2, forskolin stimulated the expression of RGS2 mRNA in a dose-dependent manner (0.01 μM to 100 μM) and reached plateau status of RGS2 up-regulation at 10 μM. Therefore, we carried out the following experiments using 10 μM forskolin. In addition, forskolin time-dependently stimulated the expression of RGS2 mRNA level, which reached the peak at 120 min (Fig. 3). Actinomycin D was employed to evaluate the contribution

of a new transcriptional control in these regulatory mechanisms. As shown in Fig. 4, actinomycin D completely reduced the expression of RGS2 induced by 10 μM forskolin.

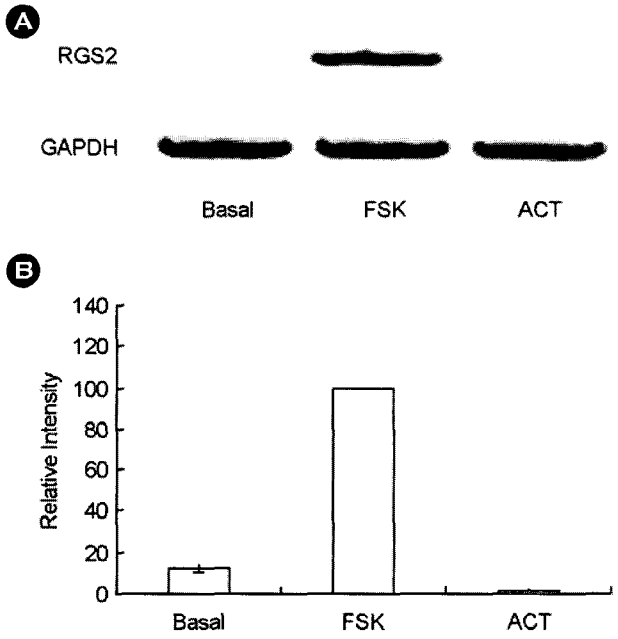

Fig. 4. Effect of transcription inhibitor actinomycin D on the expression of RGS2 mRNA in C6 cells. Qualitative **(A)** and quantitative results **(B)** of the gel electrophoresic separation of primer-specific PCR products. The columns indicate the expression rate relative to the expression of GAPDH given as mean ± SEM, n=3.

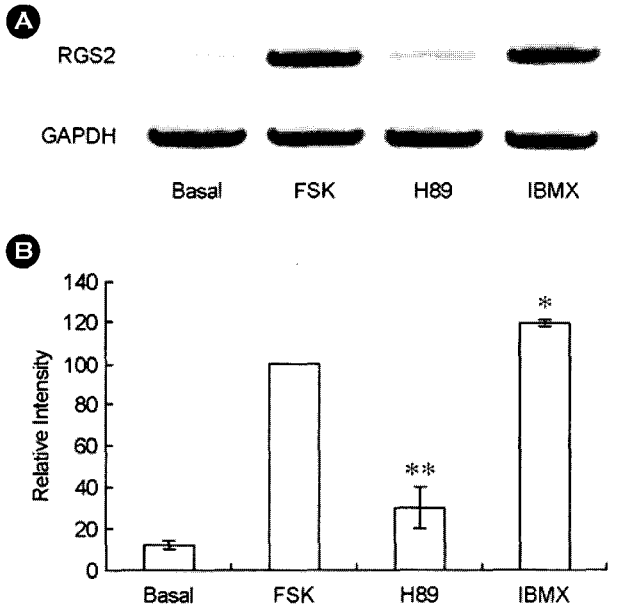

Fig. 5. Effect of PKA inhibitor H-89 and PDE inhibitor IBMX on the expression of RGS2 mRNA in C6 cells. Qualitative **(A)** and quantitative results **(B)** of the gel electrophoresic separation of primer-specific PCR products. The columns indicate the expression rate relative to the expression of GAPDH given as mean ± SEM, n=3. *<0.05 compared to the FSK-treated cells. **<0.01 compared to the FSK-treated cells.

3. The effect of kinase A inhibitor H89 on forskolin-stimulated RGS2 up-regulation

Fig. 5. shows that H89 (50 μM) significantly reversed the up-regulation of RGS2 transcripts induced by forskolin. This result suggests that the regulation of RGS2 expression is involved in the signaling of PKA. Because cAMP is a direct stimulator of PKA in astrocytes (Melcangi et al., 1995), we next examined whether IBMX, a non-selective inhibitor of phosphodiesterase (PDE), affects RGS2 up-regulation. The increase of intracellular cAMP is induced both by enhanced production (via the activation of AC activity) and by reduced degradation (via the inhibition of PDE activity) of cAMP. As expected, IBMX (1 mM) potentiated the FSK-induced the up-regulation of RGS2 mRNA to a certain but statistically significant extent. The slight effect of IBMX on the potentiation of up-regulated RGS2 mRNA is possibly due to two factors: First, IBMX is not the specific and potent PDE inhibitor. The PDE family consists of

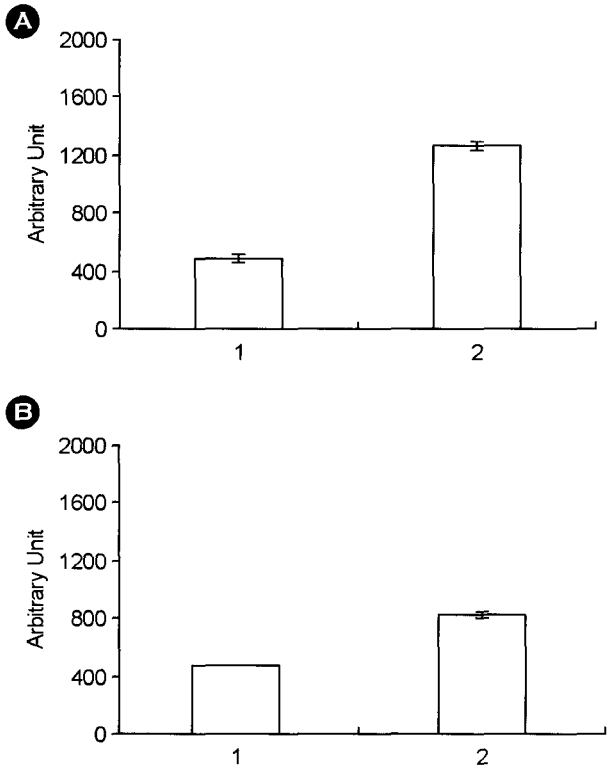

Fig. 6. Effect of forskolin on the CRE luciferase activities in C6 cells **(A)** and HEK293 cells **(B)**. The C6 cells and HEK293 cells are transfected with pCRE plasmids and treated with FSK (10 μM) for 2 hr. Values are mean ± SEM of arbitrary unit (n=3). * P<0.05 versus vehicle control (one-way ANOVA).

more than 5 members including PDE1, PDE2 and PDE5. It is recommended that both IBMX and another PDE inhibitor such as RO20-1724 are used together in cell culture system (Bayewitch et al., 1996; Rhee et al., 2000). Secondly, the extent of RGS2 mRNA's expression might be closed at an upper limit in rat C6 astrocytoma cells. These above results indicate that the cAMP-PKA pathway plays an important role in the regulation of RGS2 gene in astrocytoma cells.

4. The transcriptional control in forskolin-stimulated C6 cells

Regulation of gene transcription by cAMP classically involves the recruitment of CREB family transcription factors (Calin-Jageman et al., 2006). Especially in eukaryotes, transcriptional regulation upon stimulation of the AC signaling pathway is mediated via CREB transcriptional activity (Calin-Jageman et al., 2006; Sassone-Corsi, 1995). To investigate the role of CREB, we utilized overexpression of CRE reporter plasmid into HEK293 cells and C6 cells using luciferase reporter gene assay. As shown in Fig. 6, pretreatment with 10 μM FSK significantly activated the CRE luciferase activity in C6 cells and HEK293 cells. To determine whether CRE activity plays a crucial role in forskolin-mediated RGS2 regulation, we need to investigate the dominant-negative mutant CREB-A, which blocks DNA binding of CREB and related transcription factors (e.g. TF-1 and CREM) (Ahn et al., 1998). The study on the dominant-negative mutant remains to be evaluated in our future studies.

In conclusion, we found that RGS2, 3, 6, 9, 10, 12, 14, and 16 were expressed in rat C6 astrocytoma cells with a differential extent relative to the expression of GAPDH. Among them, the expression level of RGS2 was stimulated by 10 μM FSK in a dose- and time-dependent manner. FSK-induced stimulation of RGS2 mRNA transcription was PKA dependent and under transcriptional control. In addition, the up-regulation of RGS2 mRNA by 10 μM FSK was mediated through CREB transcriptional activity as judged by a CRE luciferase reporter gene assay. Taken together, the above results suggest that the expression level of RGS2 mRNA is up-regulated by the activation of cAMP-PKA pathway, and that RGS2 play an important role in the neuroprotective or/and neurodegerative activities of intracellular cAMP upon activation of GPCR signaling.

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