Journal of Life Science 2009 Vol. 19. No. 11. 1506~1513 ⓒJLS / ISSN 1225-9918

RGS3 Suppresses cAMP Response Element (CRE) Activity Mediated by CB2 Cannabinoid Receptor in HEK293 Cells

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Received August 4, 2009 / Accepted August 26, 2009

RGS proteins have been identified as negative regulators of G protein signalling pathways and attenuate the activity of GPCR receptors. However, information on the regulatory effects of RGS proteins in the activity of cannabinoid receptors is limited. In this study, the role of RGS proteins on the signal transduction of the CB2 cannabinoid receptor was investigated in HEK293 cells co-transfected with CB2-receptors and plasmids encoding RGS2, RGS3, RGS4 and RGS5. Treatment of cells with WIN55, 212-2, a CB2 receptor agonist, inhibited forskolin-induced cAMP response element (CRE) activity in CB2-transfected HEK293 (CB2-HEK293) cells. This inhibitory effect of WIN 55, 212-2 on CRE activity was reversed by co-transfection of CB2-HEK293 cells with RGS3, but not with RGS2, RGS4 and RGS5. However, endogenous RGS3 protein knocked down by a small interfering siRNA targeting RGS3 gene enhanced inhibition of forskolin induced CRE activity via agonist induced CB2 receptor signal transduction. These results indicate the functional role of endogenous RGS protein in cannabinoid signaling pathways and define receptor-selective roles of endogenous RGS3 in modulating CRE transcriptional responses to agonist induced CB2 receptor activity.

Key words : Cannabinoids, CB2 receptor, regulator of G-protein signaling-3, cAMP response element (CRE)

Introduction

Cannabinoid (CB) receptors belong to G protein-coupled receptors (GPCRs), that are widely dispersed in a variety of tissues [1,20]. To date, two subtypes of cannabinoid receptors have been identified by molecular cloning, CB1 and CB2 [27,28]. They are members of the seven-transmembrane (7-TM)-spanning GPCR superfamily. The identity of amino acid sequences to CB1 and CB2 receptors is relatively low (44%), but when compared to the TM domain, it is increased by 63%. The CB1 receptor is most abundant in the brain of mammals [21,30]. The human CB2 receptor has mainly been found to be expressed in immune cells, such as splenic macrophages, monocytes, B-cells, and natural killer cells, as well as in the tonsils and bone marrow [12,21,28,30]. This distribution suggests that the CB2 receptor can play a role in the immune system. This is contrary to the psychoactive properties attributed to cannabinoids which function via CB1 in the nervous system [22,31]. Cannabinoids (ligands of cannabinoid receptors) are grouped into three main classes. In the first group are endogenous ligands or endocannabinoids (eCBs) that include anandamide and 2-arachidonoylglycerol (2-AG), which have been extensively studied as known lipid transmitters [30]. The second are phytocannabinoids, consists of several compounds derived from the Cannabis plant family, most of which are bioactive. The most well-known bioactive phytocannabinoid is $\triangle^9$-tetrahydrocannabinol ($\triangle^9$-THC), which produces the psychotropic effects associated with marijuana use. Another bioactive phytocannabinoid, cannabidiol (CBD), regulates important physiological functions, such as immune responses and blood pressure [31]. These effects are achieved in the absence of psychotropic side effects, since CBD does not bind to CB1 receptors. The third group of ligands consists of synthetic compounds that act with varying degrees of efficacy and selectivity at cannabinoid receptors. For example, the non-selective agonists CP55940 and WIN55212-2 act at both CB1 and CB2 receptors, whereas JWH-015 and JWH-133 selectively activate CB2.

RGS proteins have been identified in mammals as negative regulators of G protein signaling pathways, which consist of the four components of the GPCR system (i.e., re-

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ceptors, G proteins, effectors, and RGS) [17,40]. An effort to identify new RGS proteins has extended this family to approximately 40 members. All RGS proteins have the potential of attenuating G-protein signaling by accelerating the rate of GTP hydrolysis through G protein α subunits called GTPase-accelerating protein (GAP) [7,9,10,36]. As a result of the GAP activity on Gαi/o, RGS proteins reduce maximal agonist inhibition of adenylyl cyclase [6,13,22]. RGS proteins, however, are not simply GAP [9]. Certain RGS isoforms can function as effector antagonists in G-protein signalling pathways, but others can also serve effectively with effectors [7]. Numerous studies of the specificity between RGS proteins and Gα subunits and GPCR receptors have been reported using biochemical, immunochemical, and functional methods. RGS proteins are GAPs for all Gα proteins [7,36,42]. RGS2 is a strong GAP for Gαq, while RGS4 and -5 are strong GAP for both Gαq and Gαi [16,18].

However, information on the involvement of RGS proteins in modulating the activity of CB2 cannabinoid receptors is limited. Reports indicated that RGS3 is a structurally distinct member of the RGS subfamily B with a unique long N-terminal domain. It has GAP activity for most Gαi family subunits and Gαq, but not for Gαz, Gαs, or Gα12 [37]. Studies have further identified receptor-specific effects of RGS protein action. In cotransfection studies of gonadotropin-releasing hormone (GnRH) receptor with different RGS cDNAs, only RGS3 expression suppressed GnRH-induced IP3 responses [29]. Stable transfection of a full-length antisense RGS3 cDNA in NIH 3T3 cells abolishes the expression of endogenous RGS3 protein and significantly increases MAP kinase phosphorylation induced by endothelin-1 stimulation [11]. Even though these previous studies have provided evidence for RGS-Gα and RGS-receptor specificity, very few reports exist that detail the contribution of endogenous RGS proteins to specific receptor responses. Moreover, the functional roles of endogenous RGS proteins in cannabinoid signaling are not clear.

To elucidate functional roles of specific endogenous RGS proteins in peripheral cannabinoid receptor signaling, we have used RGS2, RGS3, RGS4 and RGS5 proteins in CB2 receptor expressing HEK293 cells. We further have developed siRNA approach to specifically down regulate the endogenous RGS3 protein at the mRNA level. The results presented here define specific role of RGS3 in inhibiting agonist induced CB2 receptor signaling as evidenced by its modulation of forskolin induced CRE activity. This receptor-selective effect of RGS protein function provides new evidence for a unique targeting of RGS action to specific cellular responses to CB2 receptor signaling.

Materials and Methods

Materials

HEK293 cells were obtained from ATCC (Rockville, USA). Forskolin (FSK) and fatty acid-free bovine serum albumin (FAF-BSA) were purchased from Sigma (St. Louis, MO). The cannabinoid agonist, WIN 55,212-2, was purchased from Tocris Bioscience (Bristol, UK). PCR premix, RT premix, and the primer oligonucleotides were obtained from Bioneer Co. (Daejeon, Korea). Easy-BLUE^{TM} reagent was obtained from iNtRON Biotechnology (Seoul, Korea). Tissue culture reagents were from Life Technologies (Gaithersburg, MD).

Plasmids

RGS2, RGS3, RGS4, and RGS5 were purchased from the UMR cDNA Resource Center (Rolla, MO). RGS2 in pEGFP and RGS3 in pRCCMV were generously provided by Professor Kendall Blumer, Department of Cell Biology & Physiology, Washington University School of Medicine (St. Louis, MO). The human CB2 cDNA [28] was kindly provided by Dr. S. Munro (Cambridge, UK) [34].

Cell culture and transfection

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin, and 5% FBS. Cells were grown at 37°C and 5% CO_2 in humidified air. Twenty-four hr before transfection, a confluent were trypsinized and split into five 10-cm plates. The cells were transfected by using Fugene's Transfection Reagent (Roche) according to the manufacturer's protocol, with HA-CB2 receptor cDNA (0.2 μg/plate) alone, or co-transfected with either RGS2, RGS3, RGS4, RGS5 (2 μg/plate) or pXMD1gal (for mock DNA transfection). Fourty-eight hr later, the cells were trypsinized and re-cultured in 24-well plates, and after an additional 24 hr, the cells were assayed for the CRE luciferase reporter assay, as described below. Transfection efficiency was in the normal range of between 40-80%, as determined by staining for β-galactosidase activity [26].

siRNA preparation and transfection

Synthesized, purified and phosphorthioate-modified oli-

godeoxynucleotide were purchased from the Bioneer Co. (Daejeon, Korea). We designed an RGS3 siRNA duplex which corresponds to bases 1472 -1490, from an open reading frame of human RGS3 mRNA: 5′-TCA TGG AAA AGG ACT CGT T-dTdT-3′. The 19 nucleotides represent the target sequence in human RGS3 (AY585192.1) and dTdT is a 2-nt 3′ overhang. The antisense strand was composed of nucleotides that complement target sequence and the dTdT 3′ overhang sequence. All siRNAs were resuspended in DEPC-treated water to provide a final amount of 30 μM. In preliminary experiments, we optimized conditions for the efficient transfection of HEK293 cells using siRNA. Experiments were conducted at 48 hr post-transfection.

Extraction of total RNA

The total RNA from HEK293 cells transfected with the siRNA of RGS3 or control siRNA, were prepared by adding Easy BLUE^{TM} Reagent (iNtRON Biotechnology Co., Korea), according to the manufacturer's protocol. The total RNA solution was stored at -70°C until use.

Semiquantitative RT-PCR amplification

Semiquantitative RT reactions were carried out using a RT premix (Bioneer Co., Korea). Briefly, total RNAs (2 μg) were incubated with oligo-dT_{18} for 5 min at 70°C and cooled on ice for 3 min, and, after the addition of RT premix, the reaction mixture was incubated for 90 min at 42.5°C. The reactions were terminated for 5 min at 95°C with regard to the inactivation of reverse transcriptase. The PCR reaction was further continued using a PCR premix (Bioneer Co., Korea) with the appropriate sense and antisense primers for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense primer, 5′-CAC TCA CGG CAA ATT CAA CGG C-3′; antisense primer, 5′-CCT TGG CAG CAC CAG TGG ATG CAG G-3′), RGS3 (sense primer, 5′- GCT TGT GAG GAT TTC AAG AAG GTC-3′; antisense primer, 5′- GAT ACG TTT TTG TGC CAG GTC AAA GCA G-3′), under the following incubation conditions: a 45-sec denaturation time at 94°C, an annealing time of 45 sec at 55 to 60°C, an extension time of 45 sec at 72°C, and a final extension of 10 min at 72°C at the end of the cycles. PCR products were separated in a 1% agarose using electrophoresis (BioRad Co., Hercules, CA). The relative intensity levels were calculated using Eagle eyes Image Analysis software (Stratagene Co., La Jolla, CA). The resulting densities of the RGS2, 3, 4, 5 bands were expressed relative to the corresponding density of the GAPDH bands from the same RNA sample. GAPDH, a housekeeping gene, was used as a RNA internal standard.

CRE luciferase reporter gene assay

The assay was performed in triplicate as described [3] with minor modifications. In brief, HEK293 cells, transfected with appropriate plasmids and pCRE-luciferase reporter vector using FuGENE reagents, were seeded in 24-well plates (1×10^6/ml) and maintained for 24 hr. Cells were treated with test agents at the indicated concentration levels for various time periods. Luciferase activity was measured using a luciferase assay kit (Promega) and a microplate luminometer (Aureon, Austria).

Statistical analysis

A one-way ANOVA was used to determine the statistical significance of differences between the different values of the experimental and control groups. Data represent the means ± S.E.M. of three experiments conducted in triplicate. P values of 0.05 or less were considered to be statistically significant.

Results

WIN55,212-2 inhibited FSK-activated CRE luciferase activity in CB2-transfected HEK293 cells

Since the activation of CB2 cannabinoid receptor, which coupled to inhibitory G protein subunit Gαi, is capable of inhibiting AC activity and thus inhibit the FSK-activated CRE transcription activity [8,34], we examined the effect of agonist induced CB2 signal transduction on FSK induced CRE activity. As shown in Fig. 1, WIN55,212-2 (0.1 μM and 10 μM) significantly inhibited FSK-stimulated CRE luciferase activity in CB2 receptor-transfected HEK 293 cells, but not in non-transfected control cells.

Cannabinoid CB2 receptor-mediated luciferase activity is completely blocked by RGS3

To determine whether RGS proteins modulate forskolin stimulated CRE transcriptional activity, we co-transfected RGS2, RGS3, RGS4 or RGS5 with HA-tagged CB2 receptors in HEK293 cells and examined CRE luciferase reporter activity.

Among RGSs examined, RGS3 completely blocked the agonist induced CB2 receptor mediated inhibition of CRE transcriptional activity in HEK293 cells. However, other

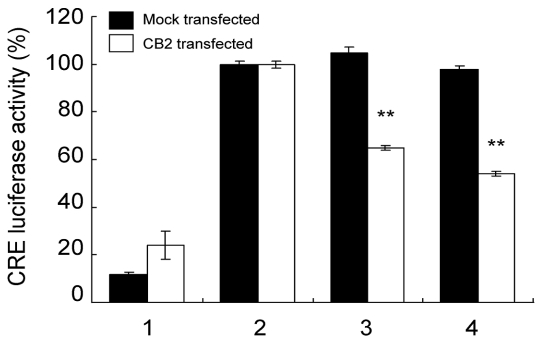

Fig. 1. WIN55,212-2 inhibited FSK (2 μM)-induced CRE luciferase activity in HEK293 cells transfected with the CB2 cannabinoid receptor (□) or in mock transfected-cells (■). HEK293 cells were transfected with HA-CB2 receptor cDNA (0.2 μg/plate), or pXMD1gal (for mock DNA transfection) using Fugene's Transfection Reagent (Roche). Fourty-eight hr later, CRE luciferase reporter assay was carried out as described in 'Materials & Methods'. Each value represents the means±SEM of three independent experiments performed in duplicate. **<0.01 vs control (FSK treatment). 1; basal; 2, FSK (2 μM); 3, FSK (2 μM) + WIN55,212-2 (0.1 μM); 4, FSK (2 μM) + WIN55,212-2 (10 μM).

RGSs including RGS2, RGS4, and RGS5, did not affect the CRE luciferase promoter activity (Fig. 2). The result was consisted with RGS plasmids from different sources either tagged with HA or GFP.

Endogenous RGS3 down regulation potentiates the inhibitory activity of WIN55,212-2 in FSK-activated CRE luciferase activity

Consistent with the known variety of endogenous receptor-G protein-effector pathways in HEK293, we determined the RGS proteins that are expressed at the mRNA levels in these cells (data not shown). The expression levels of mRNAs from transientl RGS transfected-HEK293 cells for RGSs used in the study were determined using semi-quantitative RT-PCR. Accordingly, we transfected and tested RGS2, -3, -4 and -5 genes for expression in CB2 transfected HEK293 cells by RT-PCR (Fig. 2G). All the tested RGSs were detected strongly at the mRNA levels in a similar fashion. Since RGS3 completely blocked the agonist induced CB2 receptor mediated inhibition of CRE transcriptional activity in HEK293 cells, we determined whether small interfering RNA (siRNA) targeting RGS3 gene can restore the agonist induced receptor activity. HEK293 cells were transiently transfected with siRNA targeting RGS3 or siRNA control using FuGENE transfection reagent

After 48 hr of transfection, total RNA were extracted from cells transfected with siRNA of RGS3 or control siRNA, and the level of RGS3 mRNA was determined by a semi-quantitative RT-PCR. As shown in Fig. 3, endogenous RGS3 knock down using siRNA significantly suppressed the expression levels of the RGS3 gene in a dose- dependent manner. Next, we examined the WIN55,212-2 induced receptor activity indirectly from the inhibition of FSK-stimulated CRE luciferase promoter expression levels in HEK293 cells co-transfected by siRNA of RGS3, or by the control siRNA. As Interestingly, RGS3 down regulation increased CRE luciferase reporter activity with the IC_{50} of WIN55,212-2, under the transfection of RGS3 siRNA, shifted to the left compared

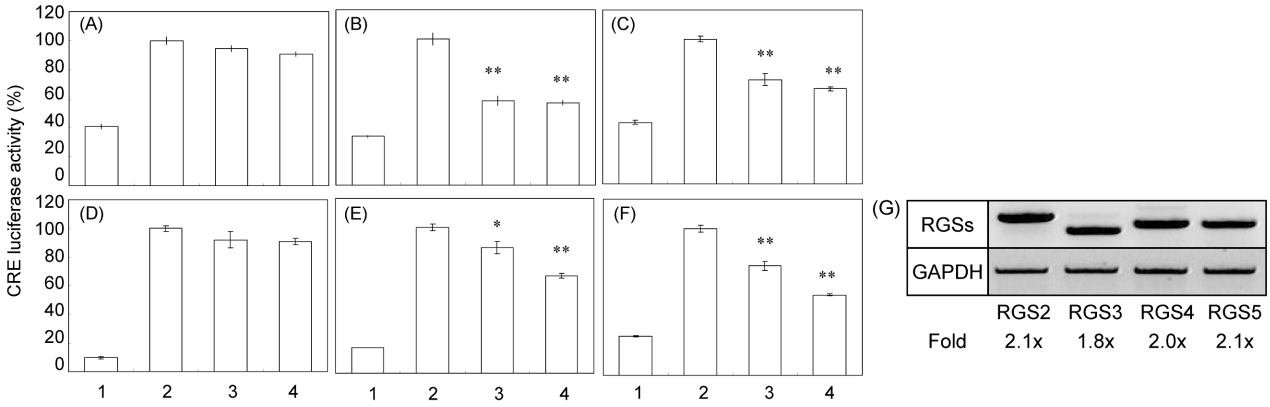

Fig. 2. Effect of overexpression of various RGSs on CB2 receptor signaling. HEK293 cells were transfected with pXMD1 (A), HA-CB2 alone (B), HA-CB2 and RGS2 (C), HA-CB2 and RGS3 (D), HA-CB2 and RGS4 (E), or HA-CB2 and RGS5 (F). The transfection of plasmids and reporter gene assay were described in 'Matierials & Methods'. Each value represents the means ± SEM of three independent experiments performed in duplicate. * $p<0.05$. The expression levels of RGS2, RGS3, RGS4, and RGS5 mRNA were not significantly different (G) as determined using semiquantitative RT-PCR. * $p<0.05$ vs control (FSK treatment). ** $p<0.01$ vs control (FSK treatment). 1; basal; 2, FSK (2 μM); 3, FSK (2 μM) + WIN55,212-2 (0.1 μM); 4, FSK (2 μM) + WIN55,212-2 (10 μM).

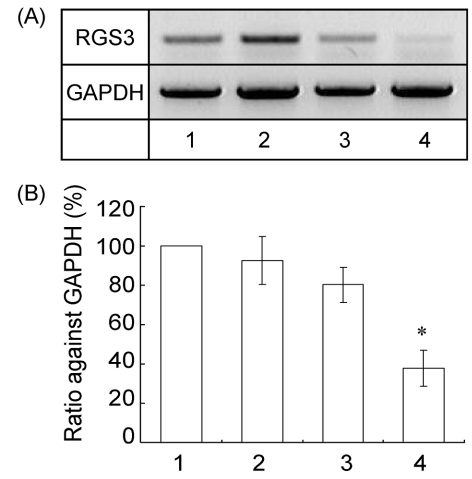

Fig. 3. Knockdown of the endogenous RGS3 gene in HEK293 cells using double-stranded siRNA. The preparation and transfection of the siRNA of RGS3 gene were described in "Materials & Methods". The extent of knockdown of the RGS3 gene was evaluated by determining the expression levels of RGS3 mRNA. The mRNA levels of RGS3, from the HEK293 cells, were determined by a semi-quantitative RT-PCR as described in "Materials & Methods". A, The bands present the results from three separate experiments (which showed similar results). B, Each value represents the means±SEM of three independent experiments performed in duplicate. * $p<$ 0.05 vs basal.

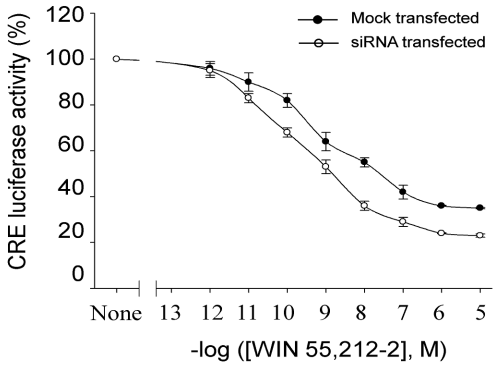

Fig. 4. Knockdown of the endogenous RGS3 gene potentiates the inhibitory activity of WIN55,212-2 on FSK (2 μM) -activated CRE luciferase activity in HEK293. The transfection of plasmids and reporter gene assay were described in 'Matierials & Methods'. The data represent the means±SEM of three independent experiments performed in duplicate.

with that of WIN55,212-2 in the control siRNA-transfected HEK293 cells (IC50s of 3.01±0.62 vs 0.31±0.15, Fig. 4). The results suggest that RGS3 protein negatively regulates agonist induced CB2 signal transductions that inhibit forskolin stimulated adenylyl cyclase activity. This in turn inhibits cAMP production and subsequent PKA mediated cAMP respose element transcriptional activity.

Discussion

This study, using CB2 receptor expressing HEK293 cells, demonstrates that endogenous RGS3 protein reduces agonist potency for the inhibition of forskolin induced CRE luciferase promoter activity. The maximal agonist response, however, was induced more by siRNA directed RGS3 proteins down regulation as evidenced by conditions of reduced CRE luciferase activation. On the other hand, the receptor activity was not affected by RGS2, RGS4 and RGS5 proteins. Although the specific Gα protein to which RGS3 mainly functions as GAPs was not determined in this study, the CB2 cannabinoid receptor mainly binds to inhibitory G proteins, Gαi, and thus, it inhibits cAMP production through the actions of AC [8,19,31,34]. The RGS3 specificity observed here could be occurring at the G protein level or may be dependent on receptor-RGS contacts (direct or indirect). Wang et, al. [39] reported that endogenous RGS3 and RGS5 in rat A-10 vascular smooth muscle cells and primary culture of aorta smooth muscle cells have selective effects on MAP kinase stimulation by muscarinic and angiotensin receptors. The observation that MAP kinase stimulation is enhanced when RGS levels are reduced suggests that there is tonic (or rapidly inducible) inhibition of these receptor responses by the endogenous RGS proteins. RGS3 has previously been shown to regulate MAP kinase responses both in overexpression and antisense knock-down studies [11].

The expression of RGS3 mRNA transcripts in normal and abnormal tissue [25,33,41], and the biochemical regulation of RGS3 protein in the signaling of some GPCRs have been well documented [4,38-40]. Recent biochemical studies suggested that RGS3 proteins selectively interact with and negatively regulate members of the $G\alpha_{i,}$ and $G\alpha_{q}$ families of G proteins [24,41-42]. RGS3 gene expression was markedly enhanced in cardiac hypertrophy, but was reduced in failing myocardium obtained from rats. This suggests that the RGS3 gene expression is highly regulated in the myocardium and that it plays an important role in regulating signaling regarding the governing of cardiac functions [41]. Wang et al. [39] using endogenous gene knockdown with RGS3 ribozyme, have shown the receptor-selective roles of RGS3 in muscarinic m3 receptor signaling.

In addition, the lysophosphatidic acid receptor-stimulated phosphorylation of endogenous ERK1 and ERK2 was markedly impaired in both RGS3 and RGS3T transfectants, demonstrating the functional ability of both RGS forms to modulate $G\alpha_i$-mediated signaling [4]. This may reflect the specificity of RGS3 toward G proteins in the $G\alpha_i$ family [4,24], and the carboxyl terminus of RGS3 comprises the structural domain for the selectivity of G proteins (i.e., Gαi, Gαs, or Gαq) [38]. RGS3 are an effective inhibitor of chemotaxis toward the lymphoid tissue chemokines stromal cell-derived factor, B lymphocyte chemoatttractants, and EBV-induced molecule 1 ligand chemokine [33]. Similar to our observations reported here, the RGS3 protein exhibits the GPCR-selective modulation of Gq/11-coupled signaling pathways [39] and other $G\alpha_{i/o}$-coupled signaling pathways [24,41]. In biochemical studies, RGS3 functioned as GAP for $G\alpha_i$ and RGS2 was more effective as a GAP for Gαq, even though there is controversy regarding such findings [5,15,17,37,39]. Therefore, the strict $G\alpha_i$-coupled CB2 receptor and the expression patterns of both RGS3 and CB2 receptor mRNA transcripts in lymphoid tissue are potential indicators of a biochemically deep relationship. Although, the biochemical mechanism by which the transfection of RGS3 proteins completely inhibited WIN-55,212-2-activated CRE luciferase activity in HEK293 cells remains to be elucidated, we can assume several factors due to previous studies [14,23,38]. These findings demonstrated that certain RGS proteins can directly and selectively interact with preferred G-protein-coupled receptors in order to modulate their signalings.

In summary, these data show that endogenous RGS3 is a negative modulator of forskolin induced CRE luciferase reporter activation by agonist stimulated CB2 receptor mediated probably through Gαi/o pathway. At the same time, endogenous RGS3 knock down reversed the potency of the agonist induced receptor inhibition on forskolin stimulated CRE transcriptional activity. Thus we demonstrate that a novel receptor selectivity of RGS3 action may be important signaling pathway in CB2 cannabinoid expressing HEK293 cells.

Acknowledgement

This research was supported by Kyungpook National University Research Fund, 2007. We wish to thank the proofreading team of KNU for their assistance.

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초록 : 캐너비노이드 수용체 CB2의 신호전달작용에 미치는 RGS3의 억제적 효과

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RGS단백질은 G 단백질 신호전달작용에 있어서 신호를 억제하는 조절단백질로서 G 단백질 매개수용체(GPCR)의 활성을 억제하는 것으로 알려졌다. 그렇지만 캐너비노이드 수용체 CB2의 활성에 있어서 RGS 단백질의 조절효과에 관해서는 지금까지 알려져 있지 않다. 그러므로 본 연구에서 우리는 RGS2, 3, 4, 5와 캐너비노이드 수용체 CB2 cDNA를 동시에 HEK293 세포주에 발현시킨 후 각 RGS 단백질의 효과를 조사하였다. CB2 단백질을 발현하는 HEK293 세포주(CB2-HEK293)에서 CB2 효현제인 WIN55,212-2는 폴스콜린으로 유도된 cAMP response element (CRE) 활성을 억제하였다. 이러한 WIN55,212-2의 CRE 억제 활성은 RGS3에 의하여 차단되었지만 RGS2, 4, 및 RGS5에서는 관찰되지 않았다. 뿐만 아니라 RGS3 small interference RNA (siRNA)를 사용하여 내인성 RGS3 단백질의 발현을 저하시키면 WIN55,212-2에 의한 폴스콜린 유도 CRE 억제활성은 더욱 증강되었다. 이상의 결과는 캐너비노이드 수용체 CB2 신호전달작용에 있어서 RGS 단백질의 기능적 역할과 특히 내인성 RGS3의 캐너비노이드 수용체 CB2에 대한 선택적 작용을 나타낸다.