学 位 論 文 の 要 旨

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学 位 論 文 名　Cyclic Adenosine 3',5'Monophosphate/Protein Kinase A and Mitogen-Activated Protein Kinase 3/1 Pathways Are Involved in Adenylate Cyclase-Activating Polypeptide 1-Induced Common Alpha-Glycoprotein Subunit Gene (*Cga*) Expression in Mouse Pituitary Gonadotroph LbetaT2 Cells

発 表 雑 誌 名　BIOLOGY OF REPRODUCTION
（巻，初頁～終頁，年）　77, 707–716, 2007

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論 文 内 容 の 要 旨

INTRODUCTION

Although the pituitary gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) are mainly controlled by the hypothalamic peptide, gonadotropin–releasing hormone (GnRH), adenylate cyclase activating polypeptide 1 (ADCYAP1) also participates in gonadotropin synthesis and released alone or in cooperation with GnRH. ADCYAP1 was first isolated from an extract of ovine hypothalamus on the basis of its ability to stimulate cAMP formation in rat pituitary cells and exerts its action via three heptahelical G-protein-linked receptors: one ADCYAP1-specific (ADCYAP1R1) receptor and two receptors (VIPR1 and VIPR2). ADCYAP1 acts predominantly via ADCYAP1R1 receptors and stimulates both inositol phosphate turnover and cAMP accumulation.

The various signal transduction pathways regulating gonadotropin subunit *Cga*, *Lhb*, and *Fshb* gene expressions are well studied and include the mitogen-activated protein kinase (MAPK) families, mitogen-activated kinase 3/1 (also known as extracellular signal-regulated kinase 1 and 2), MAPK8 (c-Jun N-terminal kinase) and MAPK14 (p38MAPK), as well as cAMP dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase pathways.

GnRH is the primary regulator of gonadotropin secretion and gene expression. Binding to its seven-transmembrane G-protein coupled receptor (GNAQ : known as Gq) stimulates an increase in inositol phosphate turnover and diacylglycerol levels, both of which ultimately activates members of the MAPK families. GnRH also couples with GNAS (known as Gs) protein and increases cAMP accumulation. Given that the binding of ADCYAP1 to the ADCYAP1R1 receptor couples with both adenylate cyclase and phospholipase C via GNAS and GNAQ proteins, it is possible that ADCYAP1 and GnRH share signal transduction systems to regulate gonadotropin gene expression.

In this study, we examined ADCYAP1 action on gonadotropin gene expression using LβT2 gonadotroph cells and described the signal transduction system, focusing on cAMP-PKA and MAPK3/1 signaling.

MATERIALS AND METHODS

Cell Culture

LβT2 cells were cultured in DMEM containing 10% FCS and 50 μg/ml streptomycin and maintained at 37°C in an atmosphere of 95% air-5% CO_2.

Cyclic AMP Accumulation

Cells were plated in 96-well plates at density of 10^4 cells/well and cultured for 72 h. Intracellular cAMP levels were measured using the cAMP enzyme immunoassay system.

Reporter Plasmid Construct and Luciferase Assay

LβT2 cells were cotransfected by electroporation with gonadotropin subunits promoter-linked luciferase vector (Luc) and pRL-TK. The activities of firefly luciferase and Renilla luciferase were measured by the Dual Luciferase Reporter Assay System with a luminometer.

Reverse Transcription and Real-Time Quantitative RT-PCR Procedure

To obtain complementary DNA (cDNA), messenger RNA (mRNA) was reversed transcribed into single stranded c-DNA. Quantification of *Cga*, *Lhb* and *Fshb* mRNA expression were obtained by real-time quantitative PCR.

Western blot analysis

Samples containing the same amount of protein were subjected to SDS-PAGE in 10% acrylamide and transferred to a polyvinylidene fluoride membrane. Following chemiluminescence detection, membranes were exposed onto X-ray film. Films were analyzed by densitometry, and the intensities of P-MAPK3/1 bands were normalized to those of T-MAPK3/1 to correct for protein loading in the case of cellular lysates.

Statistical Evaluation

Values were expressed as means ± SEM. Statistical analysis was performed using the one-way ANOVA plus the Duncan multiple range test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSION

We confirmed that treatment of LβT2 cells with 100 nM ADCYAP1 for 1 h raised intracellular cAMP levels by up to 18.9±0.03-fold compared with non-treated cells.

ADCYAP1 stimulation increased *Cga* promoter activity up to 9.06±0.17-fold, but had little effect on *Lhb* and *Fshb* promoter activities. In a manner similar to ADCYAP1, 1 mM CPT-cAMP activated the *Cga* promoter up to 10.74±1.41- fold, but had little effect on *Lhb* and *Fshb* promoter activities. At mRNA level, ADCYAP1 increased *Cga* mRNA expression up to 13.15±1.39- fold, whereas *Lhb* and *Fshb* mRNA were modestly expressed, respectively. In the experiments using the pFC-PKA plasmid vector to induce expression of constitutively active PKA, transfected cells dramatically increased *Cga* promoter activity up to 23.93±0.42 -fold compared to the pCI-neo transfected cells. These results suggest that ADCYAP1 and CPT-cAMP mainly increases the *Cga* promoter activity of the three gonadotropin subunits and that cAMP and PKA activation largely contributes to this effect.

Western blotting using anti-phospho MAPK3/1 antibody followed by treatment with 100 nM ADCYAP1 increased MAPK3/1 phosphorylation, with a maximal peak at 10 min and continued activation for 2 h after stimulation. CPT-cAMP (1 mM) also activated MAPK3/1, with a peak at 30 min. Both ADCYAP1-induced and CPT-cAMP-induced MAPK3/1 activations were completely inhibited by U0126, suggesting that ADCYAP1-induced and CPT-cAMP-induced MAPK3/1 activations were both dependent on upstream MEK kinase. In addition, both ADCYAP1-induced and CPT-cAMP-induced MAPK3/1 activations were inhibited completely in the presence of H89. Similar result was obtained by the experiment using PKI.

These results indicated that ADCYAP1-induced MAPK3/1 activation depended completely on cAMP/PKA pathways. The basal level of MAPK3/1 phosphorylation was eliminated by U0126 but not by H89 and PKI.

Involvement of PKA and MAPK3/1 pathways in ADCYAP1-induced and CPT-cAMP-induced *Cga* gene expression were examined. Inclusion of H89 or U0126 partially inhibited ADCYAP1 action on *Cga* promoter activity by similar degrees. Combining H89 and U0126 demonstrated additive inhibition of ADCYAP1-induced *Cga* promoter activity. The lack of complete inhibition suggests that both PKA and MAPK3/1 pathways each partially contribute to ADCYAP1-induced Cga gene expression. Similarly, CPT-cAMP stimulation was inhibited significantly by both H89 and U0126 but in an incomplete fashion. Thus, both ADCYAP1-stimulated and CPT-cAMP-stimulated *Cga* promoter activities depend on cAMP-PKA and MAPK3/1 pathways, but another signaling system is also involved.

We next examined whether ADCYAP1 and CPT-cAMP activate *Cre*, and examined the effect of H89 and U0126 on *Cre* luciferase reporter constructs (*Cre*-Luc). *Cre*-Luc activity was increased 4.11±0.19-fold with 100 nM ADCYAP1, and the activity was completely inhibited by H89. *Cre*-Luc activity was not inhibited in the presence of U0126, suggesting that ADCYAP1-induced *Cre* activity was completely dependent on cAMP-PKA pathways. Similar results were obtained with CPT-cAMP stimulation. *Cre* promoter activity induced by CPT-cAMP was increased 3.05±0.02-fold, and it was completely inhibited in the presence of H89 ; however, U0126 did not modify CPT-cAMP's effect on *Cre*-Luc activity. These results suggest that *Cre*-mediated transcription by ADCYAP1 and CPT-cAMP is involved in the PKA, but not the MAPK3/1 signaling pathway. MAPK3/1 signaling was not linked to the transcription factor, cAMP responsive element binding protein (CREB), and *Cre*-dependent gene expressions.

The Serum Response Element (*Sre*) is a DNA domain in the promoter region that binds the MAPK3/1-mediated transcription factor. ADCYAP1 and CPT-cAMP activated the Sre promoter by 2.03± 0.2-fold and 1.78±0.21-fold, respectively, and the effects were inhibited by U0126. Similarly, H89 completely prevented ADCYAP1-induced and CPT-cAMP-induced *Sre* promoter activities. These results suggest that *Sre*-mediated transcription by ADCYAP1 or CPT-cAMP depend totally on PKA as well as MAPK3/1 activation.

Two other pathways activated by ADCYAP1 or cAMP might contribute to *Cga* gene expression. One is a PKA-activated, *Cre*-dependent signaling pathway, and the other is a PKA-dependent, *Cre*-independent MAPK3/1 signaling pathway. The latter pathway is not prominent since activation of *Cga* gene expression by CPT-cAMP was only partially inhibited by U0126, and that CPT-cAMP-activated *Cre*-Luc activity was not modulated in the presence of U0126. CPT-cAMP-induced elevation of the *Cga* promoter activity was still present even after treatment with inhibitors of both pathways, H89 and U0126, at concentrations sufficient to suppress *Cre*-Luc and MAPK3/1 activation completely. These results suggest that an unknown mediator other than PKA and MAPK3/1 might be involved in CGA activation by cAMP.

CONCLUSION

This is a report examining the effects of ADCYAP1 on a single gonadotroph cell line, LβT2. ADCYAP1 increases the intracellular cAMP concentration, which results in increased MAPK3/1 activation in a cAMP/PKA-dependent manner in LβT2 cells. ADCYAP1 mainly increases the *Cga* promoter activity of the three gonadotropin subunits, and both PKA and MAPK3/1 seem to be involved in this regulation. However, studies with inhibitors suggested that the full activation of Cga promoters also involves an as yet unidentified pathway.