

PROTEIN KINASE A AND EPAC ACTIVATION BY cAMP REGULATES THE EXPRESSION OF GLIAL FIBRILLARY ACIDIC PROTEIN IN GLIAL CELLS

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Abstract: Cyclic adenosine monophosphate (cAMP) controls differentiation in several types of cells during brain development. However, the molecular mechanism of cAMP-controlled differentiation is not fully understood. We investigated the role of protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) on cAMP-induced glial fibrillary acidic protein (GFAP), an astrocyte marker, in cultured glial cells. B92 glial cells were treated with cAMP-elevating drugs, an activator of adenylate cyclase, phosphodiesterase inhibitor and a β adrenal receptor agonist. These cAMP-elevating agents induced dramatic morphological changes and expression of GFAP. A cAMP analog, 8-Br-cAMP, which activates Epac as well as PKA, induced GFAP expression and morphological changes, while another cAMP analog, 8-CPT-cAMP, which activates Epac with greater efficacy when compared to PKA, induced GFAP expression but very weak morphological changes. Most importantly, the treatment with a PKA inhibitor partially reduced cAMP-induced GFAP expression. Taken together, these results indicate that cAMP-elevating drugs lead to the induction of GFAP via PKA and/or Epac activation in B92 glial cells.

Key words: adenylate cyclase; Epac; GFAP; phosphodiesterase; PKA

INTRODUCTION

It was recently shown that cyclic adenosine monophosphate (cAMP)-elevating stimuli, including pituitary adenylate cyclase-activating polypeptide, lead to astrocytic differentiation of cortical precursors in rat [1]. Thus, while cAMP might play an essential role in regulating astrocytic differentiation [2-6], the signaling pathway of this cAMP-induced astrocytic differentiation remains unknown.

cAMP is a second messenger molecule that mediates signal transduction initiated by ligand binding to G-protein-coupled receptors such as adrenergic receptors. Pharmacological manipulation of cyclonucleotide phosphodiesterases (PDEs), which degrade cAMP, and adenylate cyclase (AC), which produces cAMP, provides a powerful strategy for understanding the biological processes relayed by this intracellular second messenger [7,8].

Intracellular cAMP is thought to be a widespread regulator of physiological responses [7-9]. cAMP regulates many physiological responses via different molecular players, including protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac) [3,7,8,10-12]. We have reported that cAMP regulates cell proliferation, apoptosis and inflammation via Epac and/or PKA [13,14].

In this study, we established that intracellular cAMP elevation induced morphological changes via PKA and astrocyte marker expression via PKA and/or Epac activation in B92 glial cells.

MATERIALS AND METHODS

Chemicals

Forskolin (Fsk), isobutylmethylxanthine (IBMX) and isoproterenol (ISO) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

8-(4-chlorophenylthio)-2-O-methyladenosine-3',5'-cyclic monophosphate sodium salt (8-CPT-cAMP) and 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) were purchased from Biaffin GmbH & Co KG (Kassel, Germany). H89 and tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich Japan (Tokyo, Japan), respectively. Anti-vasodilator-stimulated phosphoprotein (VASP), anti-gial fibrillary acidic protein (GFAP), anti- β actin, horseradish peroxidase (HRP)-linked anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-phospho-specific VASP (Ser157) antibody and donkey anti-mouse IgG H&L (Alexa Fluor[®] 488) were purchased from Calbiochem and Abcam (Cambridge, UK), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Wako Pure Chemical Industries, Ltd. and Invitrogen (Carlsbad, CA), respectively.

Cell culture

B92 rat glial cells were a gift from Prof. Ohno-Shosaku (Kanazawa University). The cells were cultured in DMEM with 10% FBS at 37°C in a 5% CO₂ incubator.

Actin filament and GFAP staining

To evaluate the actin cytoskeletons and GFAP expression, the cells were fixed in 3.7% (v/v) formaldehyde in Dulbecco's phosphate-buffered saline, permeabilized in 0.25% Triton X-100 and blocked with 3% normal goat serum [15]. F-actin and GFAP were visualized by TRITC-labeled phalloidin and Alexa Fluor[®] 488-labeled antibody, respectively, using an inverted fluorescence microscope EVOS (Life Technologies, Tokyo, Japan).

Elongated cell counts

Cells were photographed under a microscope, and three randomly selected fields (using a 10x objective lens) from each culture dish (n=3) were captured for quantification.

RT-PCR

Total RNA was isolated using the FastPure RNA Kit (Takara, Tokyo, Japan). cDNAs were synthesized from 1 μ g total RNA using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara). PCR-based specific gene amplification for GFAP and β actin was performed with LA Taq (Takara). The PCR conditions were 35 cycles of amplification (30 s at 94°C; 30 s at 55°C; 30 s at 72°C) or 25 cycles of amplification (30 s at 94°C; 30 s at 60°C; 30 s at 72°C) for GFAP or β actin, respectively. The following primers were used for GFAP, forward: 5'-GAT GAT GGA GCT CAA TGA CCG-3', reverse: 5'-CAG CCT CAG GTT GGT TTC ATC-3'; β actin, forward: 5'-ATG GTG GGT ATG GGT CAG AAG-3', reverse: 5'-CTG GGG TGT TGA AGG TCT CAA-3'. The PCR product were separated on a 1.9% agarose gel and stained with ethidium bromide.

Immunoblot analysis

Immunoblotting was performed as described [13]. Briefly, proteins were extracted from cells and protein concentrations were determined using a protein assay. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), followed by incubation with HRP-linked secondary antibodies (1:2000).

Statistical analysis

The data are presented as means \pm SEM from at least three independent experiments. Statistical analysis was performed by ANOVA with Dunnett's test. The results were considered statistically significant when $p < 0.05$.

RESULTS

Activator of adenylate cyclase promotes the expression of GFAP and cell morphological changes

B92 cells were treated with 1 μ M forskolin (Fsk), an activator of AC. GFAP mRNA, an astrocyte marker,

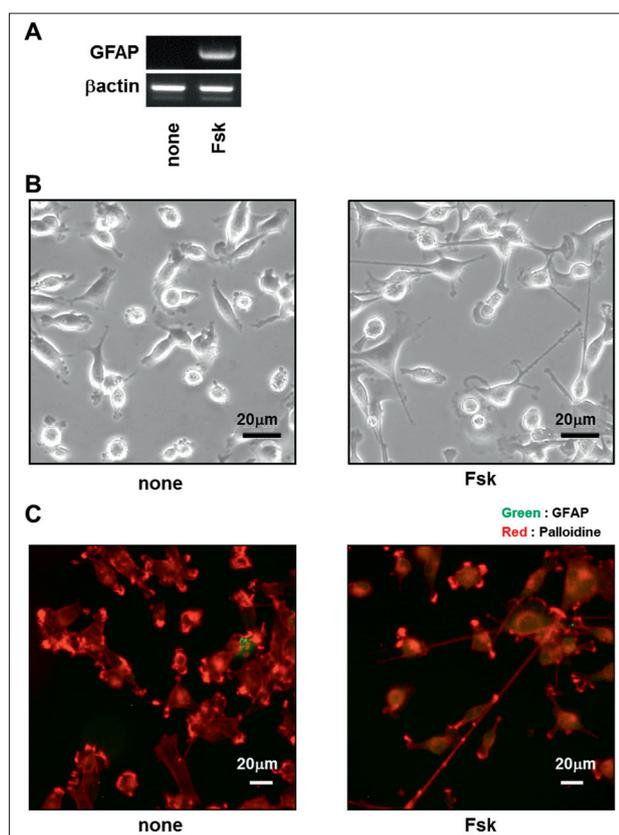


Fig. 1. Forskolin induces GFAP expression in glial cells. Changes in the expression of glial fibrillary acidic protein (GFAP) gene (A), cell morphology (B), and GFAP immunoreactivity (C) 72 h after treatment of B92 glial cells with forskolin (Fsk) (1 μ M). White or black bars indicate 50 μ m. Data is representative of at least three independent experiments.

was elevated within 72 h of Fsk stimulation in B92 glial cells (Fig. 1A and Fig. S1A). Microscopic observation revealed that Fsk regulated cell morphological changes, including elongation and the acquisition of a spindle-shape (Fig. 1B), and increased GFAP immunoreactivity in cells (Fig. 1C).

Phosphodiesterase inhibitor with/without β 2 adrenal receptor agonist promotes expression of GFAP and cell morphological changes

B92 cells were treated only with IBMX, a PDE inhibitor, or a combination of IBMX and ISO, a β 2 adrenal receptor agonist. The β 2 adrenal receptor is coupled to AC [16] and IBMX inhibits PDE, resulting in an increased intracellular level of cAMP. The treatment with IBMX (1 μ M) increased the level of GFAP gene expression (Fig. 2A and Fig. S1B). In addition, the

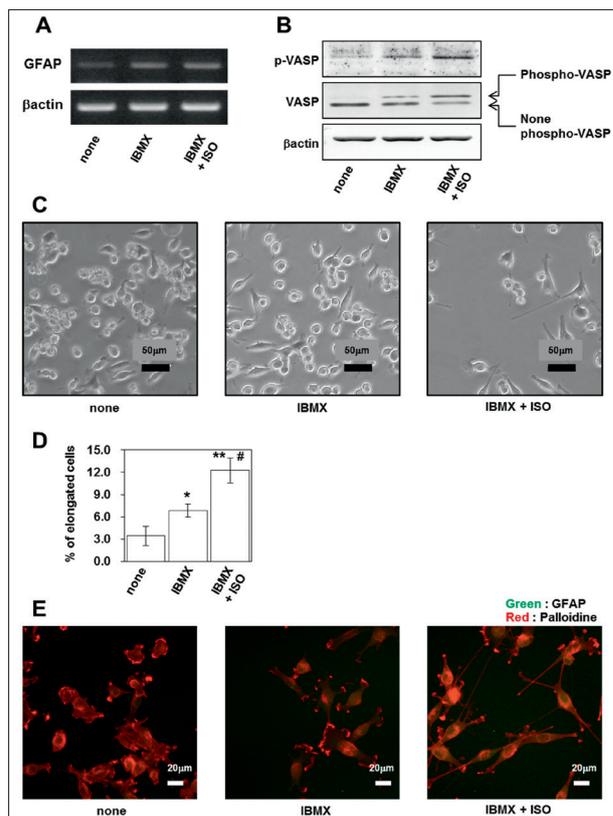


Fig. 2. IBMX and/or isoproterenol induce(s) GFAP expression in glial cells. Changes in the expression of GFAP gene (A) 72 h after treatment of B92 glial cells with only isobutylmethylxanthine (IBMX) (1 μ M) or a combination of IBMX (1 μ M) and isoproterenol (ISO) (1 μ M). Changes in the level of phospho-vasodilator stimulated phosphoprotein (VASP) (B) 30 min after treatment of B92 glial cells with only IBMX (1 μ M) or the combination of IBMX (1 μ M) and ISO (1 μ M); Changes in cell morphology (C), percentage of elongated cells (D), and GFAP immunoreactivity (E) 72 h after the treatment of B92 glial cells with only IBMX (1 μ M) or the combination of IBMX (1 μ M) and ISO (1 μ M). Black bar indicates 50 μ m, white bar indicates 20 μ m. Data is representative of at least three independent experiments. Each column represents the mean \pm SEM from three independent experiments. * P <0.05 or ** P <0.01 vs none, # P <0.05 vs IBMX.

treatment with IBMX and ISO greatly increased the level of GFAP gene expression in comparison with IBMX stimulation alone (Fig. 2A and Fig. S1B). While VASP is a critical molecule in the regulation of actin dynamics, the increase in the level of phospho-VASP expression points to cAMP elevation [16,17]. Treatment with IBMX (1 μ M) increased the level of phospho-VASP expression (Fig. 2B and Fig. S1C). Additionally, treatment with IBMX in combination with ISO greatly increased the level of phospho-VASP expression in comparison with IBMX stimulation alone

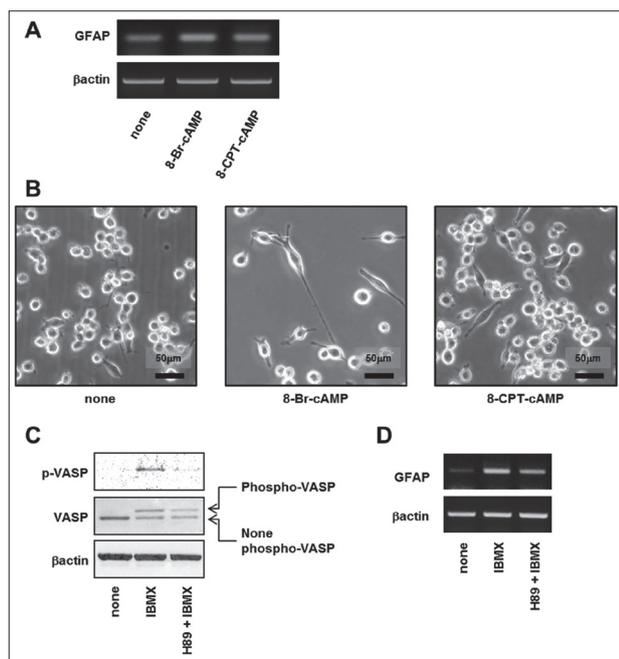


Fig. 3. cAMP analogs induce GFAP expression in glial cells. Changes in the expression of GFAP gene (A) and cell morphology (B) 72 h after treatment of B92 glial cells with 8-Br-cAMP (0.5 mM) or 8-CPT-cAMP (1 μ M). Changes in the expression of phospho-vasodilator stimulated phosphoprotein (VASP) (C) 30 min after the treatment of B92 cells with only IBMX (1 μ M) or the combination of H89 (5 μ M) and IBMX (1 μ M). Changes in the expression of GFAP gene (D) 72 h after the treatment of B92 cells with only isobutylmethylxanthine (IBMX) (1 μ M) or the combination of H89 (5 μ M) and IBMX (1 μ M). Black bar indicates 50 μ m. Data is representative of at least three independent experiments. 8-CPT-cAMP is: 8-(4-chlorophenylthio)-2-O-methyladenosine-3',5'-cyclic monophosphate sodium salt; 8-Br-cAMP is: 8-bromo-adenosine-3',5'-cyclic monophosphate.

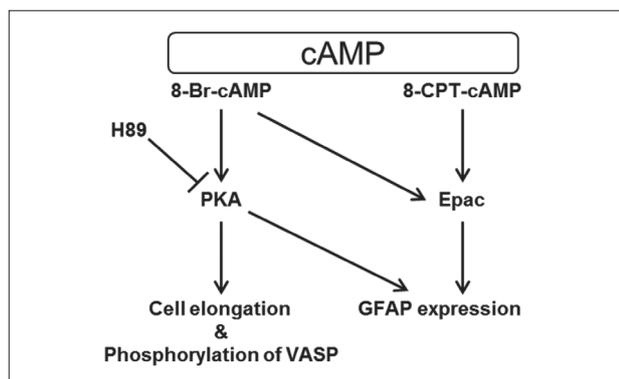


Fig. 4. A proposed model for cAMP-dependent astrocyte marker expression and morphological changes. 8-Br-cAMP is an agonist of both PKA and Epac. 8-CPT-cAMP is a specific agonist of Epac. H89 is PKA inhibitor. PKA contributes to cAMP-induced cell elongation, phosphorylation of VASP, and GFAP expression. Epac contributes to cAMP-induced GFAP expression.

(Fig. 2B and Fig. S1C). Therefore, the GFAP gene expression pattern correlated with the intracellular phospho-VASP level, indicating that astrocyte marker expression increased in an intracellular cAMP level-dependent manner. Microscopic observation showed that the cells were dramatically extended and spindle-shaped after both IBMX and ISO treatments (Fig. 2C, 2D), and that GFAP immunoreactivity increased after stimulation with IBMX, alone or in combination of IBMX and ISO in cells (Fig. 2E).

cAMP-promoted morphological changes and expression of GFAP are dependent on PKA and both PKA and Epac, respectively, in glial cells

To examine the potential signaling role of the downstream effectors of cAMP, we investigated the effects of the non-hydrolysable cAMP analogs 8-Br-cAMP and 8-CPT-cAMP on the level of GFAP gene expression and cell morphology in glial cells. Both 8-Br-cAMP and 8-CPT-cAMP upregulated the level of GFAP gene expression in glial cells (Fig. 3A and Fig. S1D), indicating that Epac contributes to GFAP gene expression. However, as can be seen in Fig. 3B, 8-Br-cAMP induced morphological changes, including elongation and spindle shape, but 8-CPT-cAMP induced very weak morphological changes compared to the 8-Br-cAMP. These results indicate that PKA, and not Epac, contributes to the morphological changes in the B92 cells.

Next, we examined whether the increased GFAP gene expression by cAMP is due to PKA activation. For this, we investigated the ability of the PKA inhibitor H89 to abrogate the IBMX-induced expression of GFAP mRNA. Pretreating cells with H89 partially blocked both PKA activation (Fig. 3C and Fig. S1E) and GFAP expression (Fig. 3D and Fig. S1F) by IBMX, indicating that cAMP-induced GFAP expression is partially dependent on PKA activation.

DISCUSSION

cAMP signal transduction plays a key role in the regulation of cell growth and development [2,3,18,19]. The elevation of intracellular cAMP activates disparate cAMP targets, including PKA and Epac. Herein we describe the role of PKA and Epac in cAMP-induced

morphological changes and increase in GFAP expression in B92 glial cells.

cAMP is an intracellular second messenger molecule that mediates many signal transduction pathways, including cell differentiation and morphological changes [2-6]. Intracellular cAMP levels are regulated by G-protein-coupled receptors, such as adrenergic receptors, adenylate cyclase, which produces cAMP, and phosphodiesterase which degrades cAMP [7,8]. In this study, we used pharmacological manipulations to increase the level of intracellular cAMP, isoproterenol (ISO) as a β 1-adrenoreceptor agonist, forskolin (Fsk) as an adenylate cyclase activator and isobutylmethylxanthine (IBMX) as a phosphodiesterase inhibitor.

Elevated cellular levels of cAMP lead to the activation of different cAMP targets, PKA and Epac. Activation of PKA by intracellular cAMP induces VASP phosphorylation. Thus, the observed increase in VASP phosphorylation pointed to increased levels of intracellular cAMP [16,17]. We used pharmacological manipulations to regulate the activities of PKA and Epac, including cAMP analogs 8-Br-cAMP as both a PKA and Epac agonist, 8-CPT-cAMP as an Epac-specific agonist and H89 as a PKA inhibitor. We demonstrated the different roles of PKA and Epac on cAMP-induced morphological changes and increased GFAP expression in B92 glial cells. Our findings are summarized in a model presented in Fig. 4.

Interestingly, PI3K and Ca^{2+} were shown to play a role in astrocytic differentiation, including GFAP expression [20-23] and morphological changes [21,22]. In our previous studies, we observed that intracellular cAMP activates tumor suppressor protein phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and, in turn, induces inhibition of cell proliferation via Akt deactivation in B92 glial cells, U87 glioma cells and osteosarcoma cells [13,14,18]. PTEN is a phosphoinositide-phosphate phosphatase specific for the 3-position of the inositol ring [24] and a key regulator of cellular PI-(3,4,5)-P3 levels. Phosphoinositide 3-kinase (PI3K) as well as PTEN is another key regulator of cellular PI-(3,4,5)-P3 (PIP3) levels. PTEN and PI3K have opposing effects on cellular PIP3 levels and consequently affect cell proliferation, survival and differentiation through various PIP3 downstream molecules, including Akt. Thus, PIP3 downstream molecule

signal pathways are negatively or positively regulated by PTEN or PI3K, respectively [25,26]. Therefore, cAMP-activated PTEN is predicted to prevent PI3K-mediated astrocytic differentiation in glial cells.

Liu et al. [19] have shown that cycloheximide induces cAMP-dependent cell differentiation in C6 glioma cells concomitantly with cell cycle arrest, leading to inhibition of cell proliferation. Differentiation to acquire specific cellular phenotypes in the developing tissue coincides with the withdrawal of precursors from the cell cycle. Therefore, it is possible that the same molecules that promote cell differentiation act as signals to inhibit proliferation. Several reports have shown that PTEN regulates multiple steps in CNS development, including astrocytic differentiation [27-31]. Although PTEN is not a classical target of cAMP action, cAMP-dependent PTEN activation has been reported in alveolar macrophages and human glioma cells [32,33]. Further studies are necessary to reveal the role of PTEN in the relationship of GFAP expression by cAMP-elevation in B92 glial cells.

CONCLUSIONS

The two families of cAMP effectors, PKA and Epac, regulate mechanisms for precise and integrated control of cAMP signaling pathways. PKA and Epac may act independently, synergistically or antagonistically in controlling specific cellular functions [16-18,34-38]. In this study, we showed that PKA and Epac contribute to cAMP-induced GFAP expression, while PKA, rather than Epac, is responsible for cAMP-dependent morphological changes. We provide insight into differentiation through PKA and Epac downstream of cAMP-induced signaling in glial cells.

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Authors' contributions: NS designed the study, conducted the experiments and prepared the manuscript. SM and NS analyzed the data. NS obtained the funding. HN, HT and AY edited the manuscript. All authors read and approved the final version of this manuscript.

Conflicts of interest disclosure: The authors do not have any conflicts of interest to declare.

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Supplementary Data

Fig. S1. Changes in expression of GFAP mRNA after the treatment with Fsk (1 μ M) (A), expression of GFAP mRNA (B) and phospho-VASP (C) after treatment with IBMX (1 μ M) or the combination of IBMX (1 μ M) and ISO (1 μ M), expression of GFAP mRNA after the treatment of 8-Br-cAMP (0.5 mM) or 8-CPT-cAMP (1 μ M) (D), expression of phospho-VASP (E) and GFAP mRNA (F) after treatment of IBMX (1 μ M) or the combination of H89 (5 μ M) and IBMX (1 μ M). Each column represents the mean \pm SEM from at least three independent experiments. *P<0.05 or **P<0.01 versus none, ##P<0.01 versus IBMX. The data can be viewed via the following link: www.serbiosoc.org.rs/sup/2016ABS0112FigureS1.tif