Research Paper

Dopamine enhances the glial endocannabinoid signaling

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Abstract Endocannabinoid system (ECS) is widely distributed throughout the body and plays important roles in the physiological functions. In this study, we investigated the effect of some neurotransmitters for the expression state of ECS-related genes of C6 cells. We recognized the significant up-regulation of phospholipase c beta 4 (PLCβ4) and down-regulation of monoacylglycerol lipase (MGLL) by dopamine (DA) treatment. The similar significant expression change of PLCβ4 and MGLL were observed by selective D1 receptor agonist treatment. Because PLCβ4 and MGLL were involved in turnover of 2-arachidonylglycerol (2-AG), it was suggested that DA might affect 2-AG productivity in C6 cells. After pre-treatment with DA, C6 cells were treated with calcium ionophore to induce the 2-AG production. The amount of 2-AG significantly increased in DA pre-treatment group. It is known that endocannabinoids act as retrograde messengers and inhibit several synaptic signal transductions. In this study, we used C6 cells as a model of astrocyte. In the synapse, astrocyte forms the tripartite synapse with presynaptic neuron and postsynaptic neuron, and is known to influence the synaptic signal transductions. If astrocytes actually respond to increased DA in the same manner as C6 cells, we suppose that the increased DA at synapse induces up-regulation of 2-AG production through activation of D1 receptors, and then the 2-AG acts on the CB1 receptors existing on the presynapse and inhibit DA release from the presynaptic ends. In conclusion, our findings suggest that the glial 2-AG signaling may act as inhibitory mechanism of synaptic DA signaling.

Key words: dopamine, endocannabinoid system, phospholipase c beta 4, monoacylglycerol lipase, 2-arachidonylglycerol

Introduction

2-arachidonylglycerol (2-AG) and N-arachidonylethanolamide (AEA) are known as major endocannabinoids[1−3]. The endocannabinoid system (ECS) consists of these endocannabinoids (2-AG and AEA), cannabinoid receptors type1 (CB1) and type2 (CB2), and enzymes involved in biosynthesis or degradation of endocannabinoids (*N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), phospholipase c beta 4 (PLCβ4), diacylglycerol lipase alpha (DAGLα), fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGLL))[4]. Endocannabinoids are produced with an activity dependent manner from phospholipid which is the component of the cell membrane (AEA is synthesized by NAPE-PLD and 2-AG is synthesized by PLCβ4 and DAGLα). Then endocannabinoids are released to the extracellular space and act on the receptors of target cells. After having acted, endocannabinoids are hydrolyzed (AEA is degraded by FAAH and 2-AG is degraded by MGLL). These ECS is widely distributed throughout the body and plays important roles in the several physiological functions[5−11]. Especially, in the central nervous system (CNS), endocannabinoids act as retrograde messengers and inhibit several synaptic transductions[12−14].

The CNS is comprised of neuron and glial cells. The

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major function of neurons is signal transduction. In the CNS, there are three types of glial cells: astrocyte, oligodendrocyte and microglia. Oligodendrocytes form myelin around axons, and contribute to saltatory conduction of action potential. Microglias have macrophage-like function, and play a role in the immune system in the CNS. It has been thought that major function of astrocytes was regulation of synapse environment and energy supply to the neurons. However, it was revealed that various neurotransmitters are released from astrocytes, and act to the neurons to regulate the neuronal functions[15].

We have been noted the expression state of ECS-related genes in the glial cell as factors to prescribe a function of ECS in the CNS. In this study, we hypothesized some neurotransmitters as the factor that affect the expression state of ECS-related genes in glial cell. We used C6 rat glioma cell line as a model of astrocytes to confirm the effect of neurotransmitters on the expression state of these genes. Furthermore, we discussed the possibility of regulation of synaptic transduction by astrocyte-derived ECS.

Materials and Methods

Reagents and chemicals

All reagents and chemicals were purchased from Sigma-Aldrich (MO, USA), and listed as follows: dopamine (DA), glutamic acid (Glu), gamma aminobutyric acid (GABA), SKF38393 (SKF), quinpirole (QNP), calcium ionophore (A23187), N-arachidonoylethanolamide (AEA), 2-arachidonyl glycerol (2-AG), methyl heneicosanoate, methanol (MeOH), acetonitrile (MeCN), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide+trimethylchlorosilane (BSTFA+TMCS). All primary antibodies were purchased from Abcam plc (Cambridge, UK) and secondary antibody was purchased from Cell Signaling Technology (MA, USA).

Cell culture and treatment

The C6 rat glioma cell line was purchased from American Type Culture Collection (VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, MO, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS, Nichirei, Tokyo, Japan) in a humidified incubator at 37°C with a atmosphere 5% CO_2. Cells were reseeded in the 6-well plates (for RNA analysis) or 100 mm dishes (for endocannabinoids analysis) at a density 5×10^4 cells/cm^2. For RNA analysis, 24 h after reseeded, C6 cells were treated by several conditions. RNA was collected at every 24 h after treatment until 72 h. For endocannabinoids analysis, C6 cells were pre-treated with or without DA for 24, 48 or 72 h. Then the culture medium was changed to FBS and DA free DMEM. C6 cells were treated with A23187 for 10 minutes, and then culture supernatant and cells were collected.

RNA isolation, PCR and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Thermo Fisher Scientific, MA, USA) and purified by chloroform/isopropanol method, followed by DNase treatment. cDNA was synthesized using Rever Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) following the manufacturer's instruction. The PCR with Platinum PCR SuperMix (Thermo Fisher Scientific, MA, USA) was performed. The amplified products and 20-bp DNA ladder were electrophoresed with agarose gel. The qRT-PCR with Platinum SYBR Green qPCR SuperMIX-UDG (Thermo Fisher Scientific, MA, USA) was performed using the ABI Prism 7900HT Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The Expression levels of each ECS-related gene were normalized against the level of hypoxanthine phosphoribosyltransferase (HPRT) in the same sample. The qRT-PCR data were shown as the value of comparative Ct method. The primers for PCR and qRT-PCR were as follows[16,17]:

CB1 5′-CAAGCACGCCAACAACACA-3′ (forward) 5′-TCTTAACGGTGCTCTTGATTGCA-3′ (reverse);

CB2 5′-TTCCCCCTGATCCCCAACGACTA-3′ (forward) 5′-CTCTCCACTCCGCAGGGCATAAAT-3′ (reverse);

MGLL 5′-ATGACCATGTTGGCCATGG-3′ (forward) 5′-CGTGCTGCAACAAATCTCTGAC-3′ (reverse);

PLCβ4 5′-GCTCTGAAACTCATTGACCGC-3′ (forward) 5′-ACGTAGCAATCTGCCATTCTCA-3′ (reverse);

FAAH 5′-CTGCCCAGCTGGTTTAAAAGA-3′ (forward) 5′-AGGACGCATACTGTTGAGAAAGG-3′

(reverse);

NAPE-PLD 5′-CTTTTGACCTCGCGGCTATT-3′ (forward)
5′-CATAAACCACCTTGGCTCATAAGC-3′ (reverse);

DAGLα 5′-AAGAATGTCACTCTCGGGATGG-3′ (forward)
5′-AGTGATGCAGACGCTGAGGAT-3′ (reverse);

HPRT 5′-GCAGTACAGCCCCAAAATGG-3′ (forward)
5′-AACAAAGTCTGGCCTGTATCCAA-3′ (reverse).

Western blot assay

C6 cells and rat brain samples were treated with cell lysis buffer to obtain total protein. The protein concentration was determined by the Quant-iT Protein Assay kit and Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Twenty micrograms of total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane (Merck Millipore, MA, USA). The membrane was incubated with the primary antibody (1 : 1,000) at room temperature for 1 h and with anti-rabbit secondary antibody (1 : 10,000) at room temperature for 1 h. The ECL detection reagent was used to determine the expression levels of the proteins.

Solid phase extraction and derivatization of endocannabinoids

The endocannabinoids in collected culture supernatants were extracted using Oasis HLB cartridge (Waters, MA, USA). The cartridge was conditioned with MeOH followed by washing with water. After loading the sample, cartridge was washed with 5% MeCN in water. The endocannabinoids were eluted from the cartridge with MeCN. Methyl heneicosanoate was added as an internal standard (IS) to eluted solution[18]. The eluted solution was evaporated, and the residue was derivatized with BSTFA+TMCS for 1 h at 60°C. Derivatized samples were analysed by gas chromatography/mass spectrometry (GC/MS).

Quantification of endocannabinoids by GC/MS

GC/MS analysis was performed on GCMS-QP2010 (Shimadzu, Kyoto, Japan) equipped with an AOC-20i auto-injector (Shimadzu, Kyoto, Japan). Analysis was performed in positive electron ionization mode. The GC/MS conditions were as follows: column, DB-5MS (30 m× 0.25 mm, i.d. 0.25 μm, Agilent Technologies, CA, USA); column oven program, 120°C for 3 min followed by an increase at 10°C/min to 300°C and keep 9 min; injector, interface and ion source temperature, 260°C, 260°C and 200°C, respectively; carrier gas and flow rate, helium at 1 mL/min[19]. MS detection was carried out in selected ion monitoring mode using *m/z* values of 432, 103 and 129 for 2-AG, *m/z* values of 404, 85 and 116 for AEA and *m/z* values of 340 for IS. Eight-point calibration curves were constructed for 2-AG and AEA in the concentration range of 50–1,000 ng/mL (r^2=0.99 and r^2=0.99, respectively). In addition, the final concentration of these endocannabinoids were standardized by total protein concentration of cells derived from the same culture dish.

Statistical analysis

All data were shown as the mean±standard error (SE). A one-way analysis of variance followed by Tukey–Kramer method was used to compare the significance of difference among all groups. Results were considered significant at $^*p<0.05$ and $^{**}p<0.01$.

Results

Expression change of ECS-related genes by neurotransmitter treatment

The expression state of ECS-related genes in C6 cells were confirmed by PCR method. As the result, the expression of CB1, MGLL, PLCβ4, FAAH, NAPE-PLD and DAGLα were detected, but the expression of CB2 was not clear in C6 cells (Fig. 1A). We examined the effect of neurotransmitter for expression state of ECS-related genes. C6 cells were treated with 100 μM DA, Glu and GABA. Then total RNA was collected at every 24 h after treatment until 72 h. As the results of qRT-PCR, significant up-regulation of PLCβ4 and significant down-regulation of MGLL were observed by 100 μM DA treatment (Fig. 1B). These expression changes continued after DA treatment from 24 to 72 h. Moreover, expression states of other ECS-related genes were not changed by DA treatment (Fig. 1B). On the other hand, expression states of all ECS-related genes were not affected by Glu and GABA treatment (Fig. 1B). In addition, LDH assay was performed to confirm influence on cell viability by neurotransmitter treatment, and significant changes were not observed among all conditions (data not

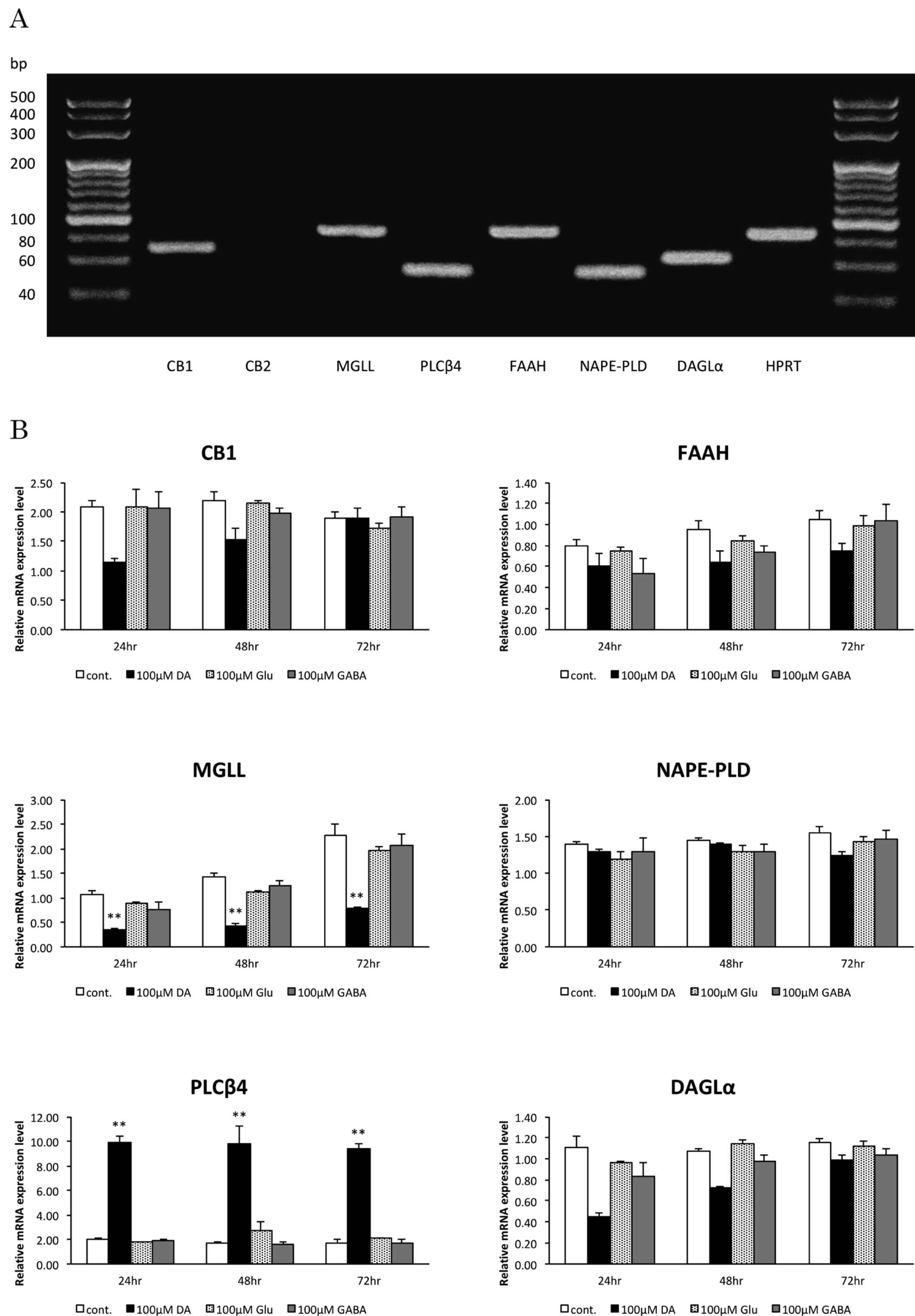

Fig. 1. Effect of neurotransmitter for expression of ECS-related genes.

(A) The expression state of ECS-related genes in C6 cells were confirmed by PCR methods. The amplified products and 20-bp DNA ladder were electrophoresed with agarose gel. The each predicted amplicon size was as follows: CB1, 68 bp; CB2, 369 bp; MGLL, 87 bp; PLCβ4, 54 bp; FAAH, 87 bp; NAPE-PLD, 53 bp; DAGLα, 63 bp; HPRT, 85 bp. (B) C6 cells were treated with 100 μM DA, Glu and GABA for 0, 24, 48 and 72 h. The expression levels of each ECS-related gene were normalized against the level of HPRT in the same sample. The expression levels of each ECS-related genes in C6 cells treated for 0-h was set at 1.00. Data were shown as folds of expression level at 0-h. All results were showed as mean±SE (n=3). $^{**}P<0.01$, compared with control sample (cont.).

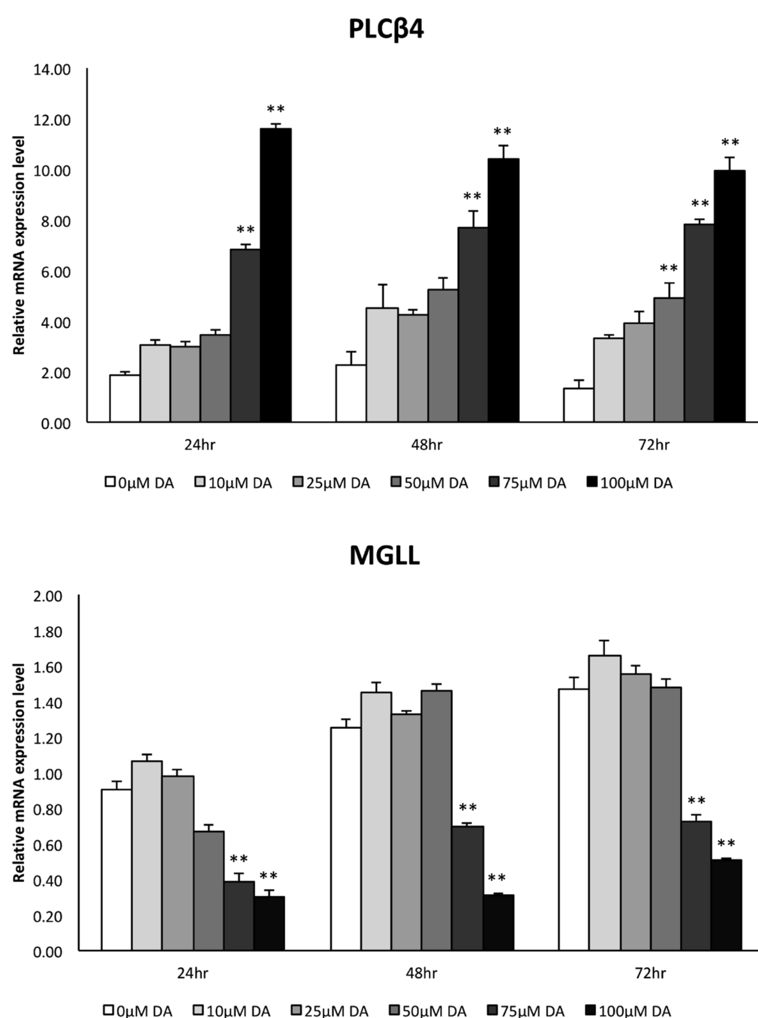

Fig. 2. DA dose dependence for expression change of PLCβ4 and MGLL.
C6 cells were treated with 0, 10, 25, 50, 75 and 100 μM DA for 0, 24, 48 and 72 h. The expression levels of PLCβ4 and MGLL were normalized against the level of HPRT in the same sample. The expression levels of PLCβ4 and MGLL in C6 cells treated for 0-h was set at 1.00. Data were shown as folds of expression level at 0-h. All results were showed as mean±SE (n=3). $^{**}P<0.01$, compared with 0 μM DA.

shown). From these results, next we performed more detailed examination for expression change of PLCβ4 and MGLL by DA treatment. C6 cells were treated with 0, 10, 25, 50, 75 and 100 μM DA to reveal DA dose dependency for expression change of PLCβ4 and MGLL. C6 cells were treated with DA for 0, 24, 48 and 72 h, and total RNA were collected at each time point. For up-regulation of PLCβ4 expression, clear DA dose dependency was observed (Fig. 2), and it was reconfirmed that up-regulation of PLCβ4 was continued until 72 h. Furthermore, clear DA dose dependency and continuity of expression change were observed in down-regulation of MGLL (Fig. 2).

DA receptor selectivity for expression change of PLCβ4 and MGLL

The DA receptors are classified in D1-like family and D2-like family. D1-like DA receptors consist of D1 and D5 subtypes, whereas D2-like DA receptors consist of D2, D3 and D4 subtypes. Next we investigated which DA receptor subtypes were involved in up-regulation of PLCβ4 and down-regulation of MGLL. By western blotting analysis, expression of D1, D3 and D4 receptors were detected in C6 cells (Fig. 3A). By using the selective agonists for D1-like DA receptor or D2-like DA receptor, we investigated involvement of DA receptor subtypes in the expression change of PLCβ4 and MGLL. SKF and QNP were used as selective D1-like DA receptor agonist and selective D2-like DA receptor agonist, respectively. C6 cells were treated

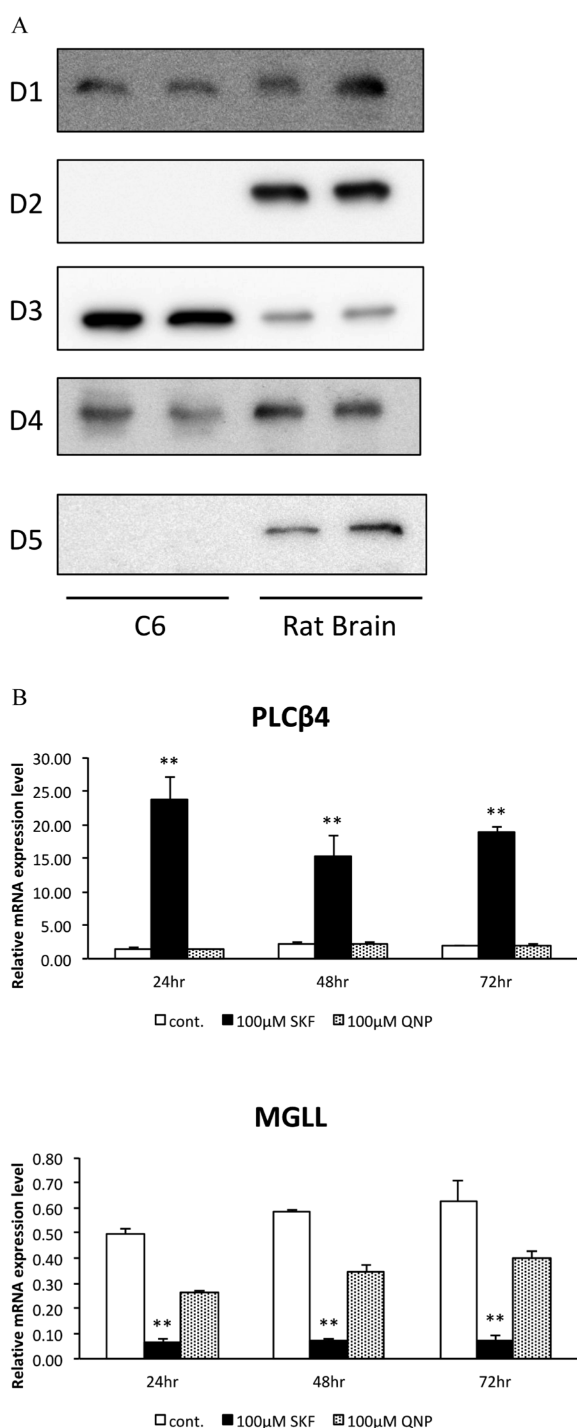

Fig. 3. DA receptor selectivity for expression of PLCβ4 and MGLL mRNA.
(A) The expression state of DA receptors in C6 cells and Wistar rat whole brain (for positive control). Immunoblots were performed with whole cell extract (n=2). Antibodies to detect D1 (ab81296), D2 (ab85367), D3 (ab155098), D4 (ab135978) and D5 (ab181623) were used. (B) C6 cells were treated with 100 μM SKF or QNP for 0, 24, 48 and 72 h. The expression levels of PLCβ4 and MGLL were normalized against the level of HPRT in the same sample. The expression levels of PLC β4 and MGLL in C6 cells treated for 0-h was set at 1.00. Data were shown as folds of expression level at 0-h. All results were showed as mean±SE (n=3). $^{**}P<0.01$, compared with control sample (cont.).

with 100 μM SKF or QNP, and total RNA collected at every 24 h after treatment until 72 h. As the result, significant up-regulation of PLCβ4 and down-regulation of MGLL were observed by 100 μM SKF treatment (Fig. 3B), and these expression changes continued until 72 h. On the other hand, any significant changes were not observed in PLCβ4 and MGLL expression by QNP treatment (Fig. 3B). In addition, LDH assay was performed to confirm influence on cell viability by SKF or QNP treatment, and significant changes were not observed among all conditions (data not shown).

Effect of DA for 2-AG turnover in C6 cells

PLCβ4 and MGLL were enzymes involved in 2-AG turnover. Therefore, it was suggested that production ability of 2-AG in C6 cells changed by intense DA exposure. Next we investigated whether 2-AG production and release in C6 cells were induced by DA treatment. After pre-treated with 100 μM DA for 24, 48 or 72 h, C6 cells were treated with calcium ionophore A23187 for 10 min, and then culture supernatants and cells were collected. The collected culture supernatants and cells were used for quantification of endocannabinoids and protein, respectively. In addition, C6 cells that were pre-treated with 0 μM DA for 24, 48 or 72 h were used for the control samples. Endocannabinoids were extracted from collected culture supernatants by solid phase extraction, and analyzed as trimethylsilyl (TMS) derivatives. In Fig. 4A, EI mass spectra from TMS derivatives of standard 2-AG and AEA were shown. In the control samples, 2-AG concentrations induced in culture supernatants by A23187 treatment were 32.3±10.0, 34.4±1.1 and 38.8±0.2 ng/mg protein at each time point (Fig. 4B). On the other hand, 2-AG concentrations in the DA pre-treated samples were 43.6±9.8, 49.9±1.0 and 91.7±7.8 ng/mg protein at each time point (Fig. 4B). In the 24-h pre-treated samples, no significant difference was observed in the 2-AG concentration between control and DA samples. However, in the 48- and 72-h pre-treated samples, 2-AG amount were significantly higher in the DA samples than control samples ($P<0.05$ (at the 48-h samples) and $P<0.01$ (at the 72-h samples)). On the other hand, AEA was not detected in all samples.

Discussion

The endocannabinoid system acts as the regulatory mechanism of synaptic signaling. Glial cells particularly astrocytes, also exist on synapses and form a tripartite synapse

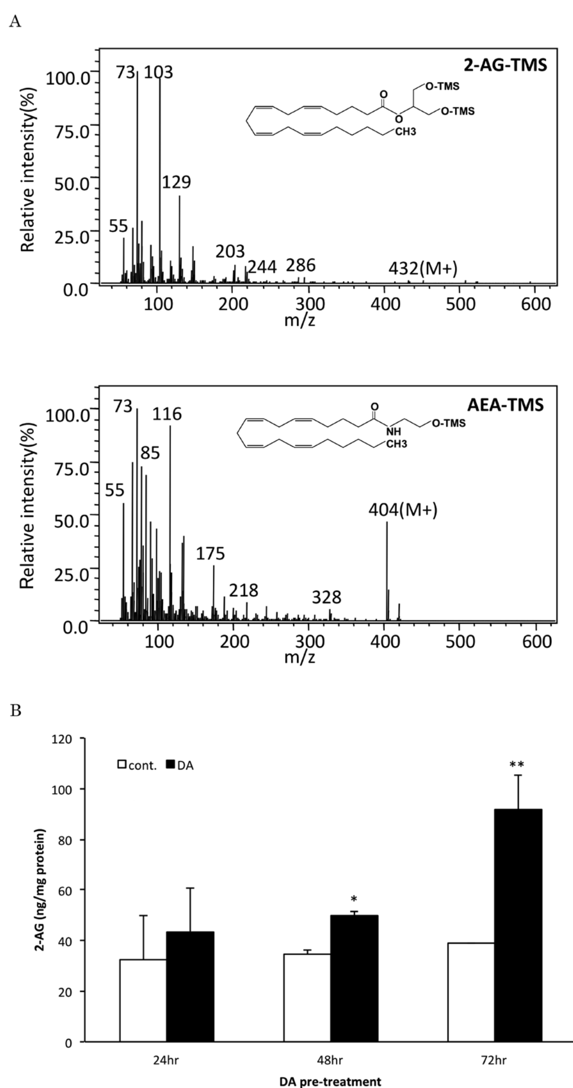

Fig. 4. Effect of DA pre-treatment for 2-AG turnover.
(A) The electron impact mass spectra obtained from TMS derivatives of standard 2-AG and AEA. (B) Extracellular 2-AG amount induced by calcium ionophore A23187 treatment. C6 cells were pre-treated with 0 or 100 μM DA for 24, 48 and 72 h. Then cells were treated with A23187 for 10 min. Culture supernatants were collected for GC/MS analysis. Samples from pre-treated with 0 μM DA were used control (cont.). All results were showed as mean±SE (n=3). $^{*}P<0.05$ and $^{**}P<0.01$, compared with cont.

with neurons. It is known that astrocytes play a role in the regulation of synapse functions. Therefore, we used C6 cells as a model of astrocytes to confirm the effect of neurotransmitters on the expression state of ECS-related genes in these cells, and discussed the possibility of regulation of synaptic transduction by astrocyte-derived ECS. The expression of ECS-related genes except CB2 in C6 cells suggests that these cells have sufficient ECS function. It is known that the distribution of CB1 and CB2 is distinctly different, CB1 is mainly found in the central nervous system, whereas CB2 is mainly found in peripheral immune cells. Furthermore, CB2 is reported slightly expressed in microglia but not in astrocytes[20,21]. Therefore, the absence of CB2 expression in C6 cells as a model of astrocytes is consistent with these facts.

We confirmed that only highly-concentrated (100 μM) DA treatment induced up-regulation of PLCβ4 and down-regulation of MGLL. Moreover, it was suggested that the expression changes of PLCβ4 and MGLL were DA-specific, because these expression changes were found to be dose-dependent on DA treatment. On the other hand, highly-concentrated (100 μM) Glu or GABA treatment did not affect the expression state of PLCβ4 and MGLL. In another preliminary study, although C6 cell treated with 1 and 10 μM Glu or GABA for 0, 24, 48 and 72 h, there were not significant changes in the expression state of PLCβ4 and MGLL (data not shown). These results also supported the DA-specificity for expression change of PLCβ4 and MGLL.

DA receptors are classified into D1-like receptor and D2-like receptor, and their actions conflict with each other due to the difference in the coupled G-proteins. Therefore, in order to confirm the receptor selectivity for expression change of PLCβ4 and MGLL with DA treatment, SKF and QNP were used as selective D1-like receptor agonist and selective D2-like receptor agonist, respectively. The significant expression change of PLCβ4 and MGLL by SKF treatment similar to that of DA treatment suggested that the activation of D1-like receptors was involved in the expression change of these genes by DA treatment. We assumed as follow that signal transduction mechanism that contributes to up-regulation of PLCβ4 via the activation of D1-like receptor by DA treatment. The activation of D1-like receptor induces activation of adenylate cyclase, because this receptor is coupled with Gs. The concentration of intracellular cyclic AMP (cAMP) is risen by activated adenylate cyclase, and as the result protein kinase A (PKA) is activated. Activated PKA is known to phosphorylate various enzymes and transcription factors. It is suggested that up-regulation of PLCβ4 by DA treatment is involvement of these cAMP dependent pathways. On the other hand, amount of MGLL mRNA was down-regulated by DA treatment. Generally, it is thought that decrease of mRNA amount is derived from inhibition of transcription by transcriptional control element or facilitation of mRNA degra-

dation as posttranscriptional regulation. However, further studies are required to reveal what kind of mechanism participates in the down-regulation of MGLL mRNA in DA-treated C6 cells. The enzymatic hydrolysis to arachidonic acid and glycerol of 2-AG is performed mainly by MGLL[19]. Because it is reported that overexpression of MGLL is involved in decrease of 2-AG amount[22] and suppression of MGLL mRNA by RNAi results in enhancement of 2-AG signaling[23], MGLL is thought to play an important role in control of 2-AG amount. Therefore, it is suggested that 2-AG production is induced by the down-regulation of MGLL mRNA by DA treatment.

Both PLCβ4 and MGLL are enzymes involved in metabolic turnover of 2-AG. Consequently, next we investigated the influence of DA on 2-AG metabolic turnover in C6 cells. Generally, it is known that endocannabinoid production and release are triggered by increase in intracellular calcium ion[19]. Therefore, A23187 was used as a calcium ionophore to induce production of 2-AG by experimentally increase the concentration of intracellular calcium ion in C6 cells. Using this experimental model, we confirmed whether the expression changes in ECS-related genes induced by DA treatment were associated with the changes in 2-AG productivity. As the result, it was revealed that 2-AG production of C6 cells were induced by DA pre-treatment. Furthermore, the influence of DA was significantly increased in proportion to pre-treatment period. From these results, it is suggested that 2-AG production ability of C6 cells changes only when cells are exposed to highly-concentrated DA for a long term. Because D1 receptor is coupled with not only Gs but also Gq, activated D1 receptor causes the activation of PLCβ family. Activated PLCβ4 hydrolyzes phosphatidylinositol bisphosphate, which constitute cell membrane, to diacylglycerol (DG) and inositol trisphosphate (IP3), and subsequently DG is converted into 2-AG by DAGLα. On the other hand, IP3 activates IP3 receptor of endoplasmic reticulum and thereby intracellular calcium concentration rises. As the result, it seems that production of 2-AG of DA-treated C6 cells was significantly increased by synergistic effect of change of mRNA expression state and condition of intracellular calcium.

Because there is much more amount of 2-AG than that of AEA in the CNS[24], it is thought that main endocannabinoid affecting the function of the CNS is 2-AG. We identified the significant increase of 2-AG production only when C6 cells were exposed to highly-concentrated DA for a long term. The brain reward system is one of the functions of CNS. In this system, neurons are mainly projected from ventral tegmental area to nucleus accumbens or prefrontal cortex, and DA acts as main neurotransmitter to communicate with other neurons. Generally, this nervous system is activated when desires are satisfied[25]. Then the DA release is increased and thereby the individual gets pleasure. However, it is known that brain reward system is directly activated by addictive drugs[26]. Repeated use of these drugs stimulates the brain reward system for a long period and produces undesirable changes including dependence and addiction. The amount of DA around synapse is known to abnormally increase by the effect of some addictive drugs[27]. The abnormality of dopaminergic neuron is occured by repeated abuse of these drugs. As the result, it is thought that drug dependence and addiction are produced by abnormality of DA signaling.

If the phenomena that we confirmed in this study reappear in vivo, it seems that 2-AG production of glial cells existing around synapse is significantly induced by abnormality of DA signaling. In the CNS, it is known that 2-AG retrogradely release from postsynapse to presynapse, act on the cannabinoid receptor exist in presynapse, and as the result, neurotransmission is suppressed[12–14]. In the synapse, astrocyte forms the tripartite synapse with presynaptic neuron and postsynaptic neuron, and is known to influence the synaptic signal transductions. Therefore, 2-AG derived from glial cells may show inhibitory effects for DA signaling. In conclusion, it is suggested that ECS derived from glial cells competes with effect of addictive drugs.

Acknowledgements

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Conflict of Interest

There are no conflict of interest relevant to this article.

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