



Comprehensive gene expression profiling of human astrocytes treated with a hepatic encephalopathy-inducible factor, alpha 1-antichymotripsin

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ARTICLE INFO

Keywords:

Astrocyte
Alpha 1-antichymotripsin
Arginine vasopressin
GPCR
cAMP
Adenylate cyclase

ABSTRACT

Astrocytes are major glial cells that play a critical role in brain homeostasis. Abnormalities in astrocytic function, such as hepatic encephalopathy (HE) during acute liver failure, can result in brain death following brain edema and the associated astrocyte swelling. Recently, we have identified alpha 1-antichymotripsin (ACT) to be a biomarker candidate for HE. ACT induces astrocyte swelling by upregulating aquaporin 4 (AQP4); however, the causal connection between these proteins is not clear yet. In this study, we utilized a microarray profile to screen the differentially expressed genes (DEGs) in astrocytes treated with ACT. We then performed Gene Ontology, REACTOME, and the comprehensive resource of mammalian protein complexes (CORUM) enrichment analyses of the identified DEGs. The results of these analyses indicated that the DEGs were enriched in pathways activating adenylate cyclase (AC)-coupled G protein-coupled receptors (GPCRs) and therefore were involved in the cyclic adenosine monophosphate (cAMP) signaling. These results indicate that ACT may act as a ligand of Gs-GPCRs and subsequently upregulate cAMP. As cAMP is known to upregulate AQP4 in astrocytes, these results suggest that ACT may upregulate AQP4 by activating AC GPCRs and therefore serve as a therapeutic target for acute HE.

1. Introduction

Hepatic encephalopathy (HE) is a major clinical complication in patients with severe liver disease and refers to the reversible neuropsychiatric disorder observed in acute liver failure (ALF) [1,2]. Rapidly progressive HE in patients with ALF is a clinical syndrome associated with cerebral edema; it leads to astrocyte swelling/brain edema, increased intracranial pressure, brain herniation, and ultimately to death. Therefore, cytotoxic brain edema, which principally occurs due to astrocyte swelling, is the major neuropathological finding in ALF [3]. Astrocytes are a major class of glial cells that play a critical role in maintaining the homeostasis of the central nervous system (CNS), and the only CNS cells that undergo rapid changes in volume during brain edema [3]. Aquaporins are a family of integral membrane proteins, which are mainly involved in the transport of water between cells [4]. Many aquaporin homologs are expressed in the CNS [4]; however, the main aquaporin expressed by astrocytes is aquaporin 4 (AQP4). AQP4 has been strongly implicated in the development of brain edema [3,4] as

brain edemas induced by water intoxication or ischemic stroke have been shown to be reduced in AQP4 null mice [5,6].

In a previous study, we identified alpha 1-antichymotripsin (ACT), referred to as SERPINA-3, as a potential causative factor for cerebral edema following acute HE and showed that ACT exerted growth-inhibitory and cytotoxic effects on astrocytes [7]. In addition, the expression of AQP4 was found to be increased following ACT treatment, and these effects of ACT were enhanced by arginine vasopressin (AVP) [7]. However, the causation between the effects of ACT and AVP and the expression of AQP4 has remained unclear. Therefore, this study aimed to clarify the potential mechanisms whereby ACT, AVP, and the combination of ACT and AVP effect AQP4 expression in cultured astrocytes. Toward this end, we utilized a microarray profile to screen differentially expressed genes (DEGs) in astrocytes after treatment with ACT and then performed various enrichment analyses of the identified DEGs, including Gene Ontology (GO) [8], CORUM (the comprehensive resource of mammalian protein complexes) [9], and REACTOME gene sets [10].

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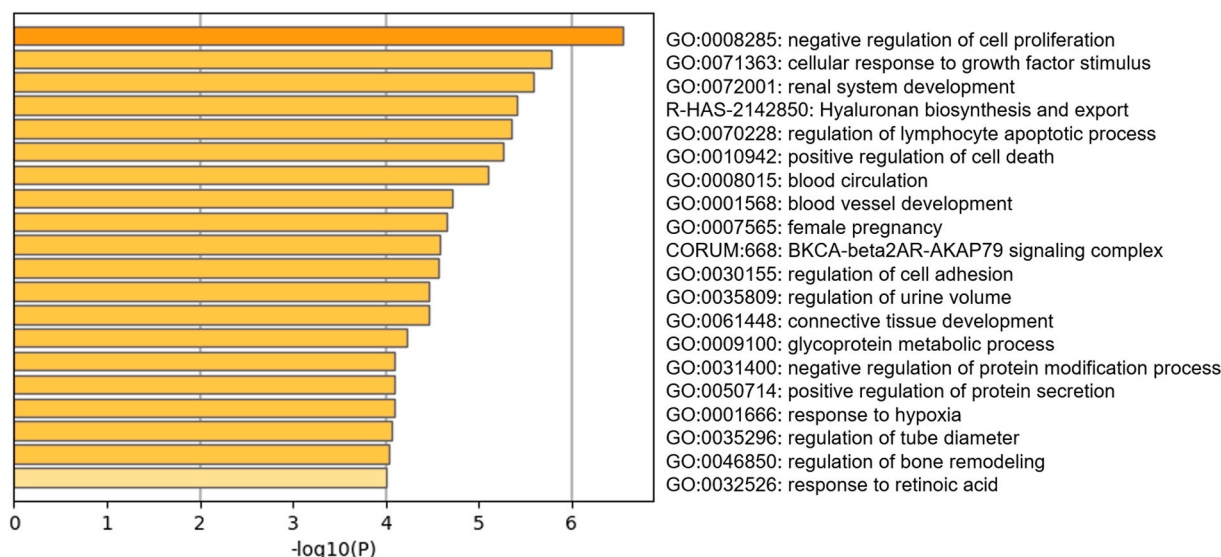


Fig. 1. Heatmap of the enriched terms among the input gene lists upon ACT treatment, colored by p -values. GO biological process, CORUM, and REACTOME gene sets were selected.

The gene functional enrichment analyses indicated that these DEGs were enriched in the cyclic adenosine monophosphate (cAMP) signaling pathway by activating adenylyl cyclase (AC)-coupled (i.e., Gs-coupled) G protein-coupled receptors (GPCRs). The 16 human $G\alpha$ subunit genes are classified into four families (Gs, Gi/o, Gq/11, and G12/13), and the members of each family interact with different effector molecules to produce distinct cellular responses [11,12]. Astrocytes express various GPCRs that can be activated by neuropeptides, neurotransmitters, and neuromodulators to up- or down-regulate cAMP and Ca^{2+} levels in cells [13,14]. The expression and function of AQP4 are subject to regulation by the cAMP signaling pathway. In fact, prolonged treatment with the cAMP analog dbcAMP has been shown to upregulate AQP4 in cultured astrocytes [15]. Therefore, our results indicate that ACT may act as a ligand of Gs-coupled GPCRs. We discuss the relationship of the interaction between cAMP and Ca^{2+} , which is known to be triggered by AVP treatment, and the possibility that ACT might serve as a therapeutic target for acute HE.

2. Materials and Methods

2.1. Cell line and culture conditions

A human astrocyte cell line was purchased from the American Type Culture Collection (CRL-8621TM, SVG p12; ATCC, Manassas, VA, USA) and maintained in ASF104 N medium (Ajinomoto, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, USA) or with 10% dialyzed FBS with an undetectable level of AVP (Biological industries, USA). Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 -containing air [7].

2.2. Treatment (cell stimulation)

The human astrocyte cell line was cultured in 24-well plates and then treated with ACT (0.5 mg/ml) for 48 h [7].

2.3. Identification of differentially expressed genes (DEGs)

Samples for the DNA-profiling studies (two biological replicates per cell line) were processed using the Agilent microarray (Agilent Technologies Inc., Japan) according to the company's standard procedures. The parameter settings for differential gene expression were as follows: p -values < .05; Fold change (FC) \geq 1.5.

2.4. Enrichment analysis

The enrichment analysis was conducted using the online tool Metascape (<http://metascape.org>) [16], which employs the standard accumulative hypergeometric statistical test to identify ontology terms. In our study, the GO biological process [8], CORUM complexes [9], and REACTOME gene sets [10] were selected to conduct the enrichment analysis. The p -values were calculated for each term, and only statistically significant data were retained. Data were considered statistically significant if the p -values were < .01.

2.5. Clustering enriched terms

Metascape was used to cluster all the resultant GO terms according to the similarities between them. Several related terms were clustered into one group, and the most enriched GO term was chosen as the representative term of the group. The overlapping nature of GO terms renders many of these terms redundant, hampering the identification of the non-redundant processes in the enrichment analysis. However, by clustering all resultant GO terms, Metascape can absorb redundancies and provide a representative term per cluster [16].

2.6. Protein-protein interaction (PPI) network and MCODE analysis

The PPI among the identified DEGs were extracted and visualized using Metascape by applying a mature complex identification algorithm called MCODE [17]. Proteins in a network are prone to forming molecular complexes; this algorithm can extract these complexes by labeling them as MCODE complexes. It then explores the function of each MCODE complex by identifying the three most enriched GO terms, thereby enabling the identification of the functional units in each PPI network.

3. Results and discussion

3.1. Enrichment analysis

After the human astrocytes were treated with ACT for 48 h, DNA microarray analysis identified 1550 genes as the DEGs according to the selection criteria specified in the "Materials and Methods" section (adjust p -value .001, p -value .05, FC1.5). Enrichment analysis indicated that the enriched GO terms after ACT treatment included negative

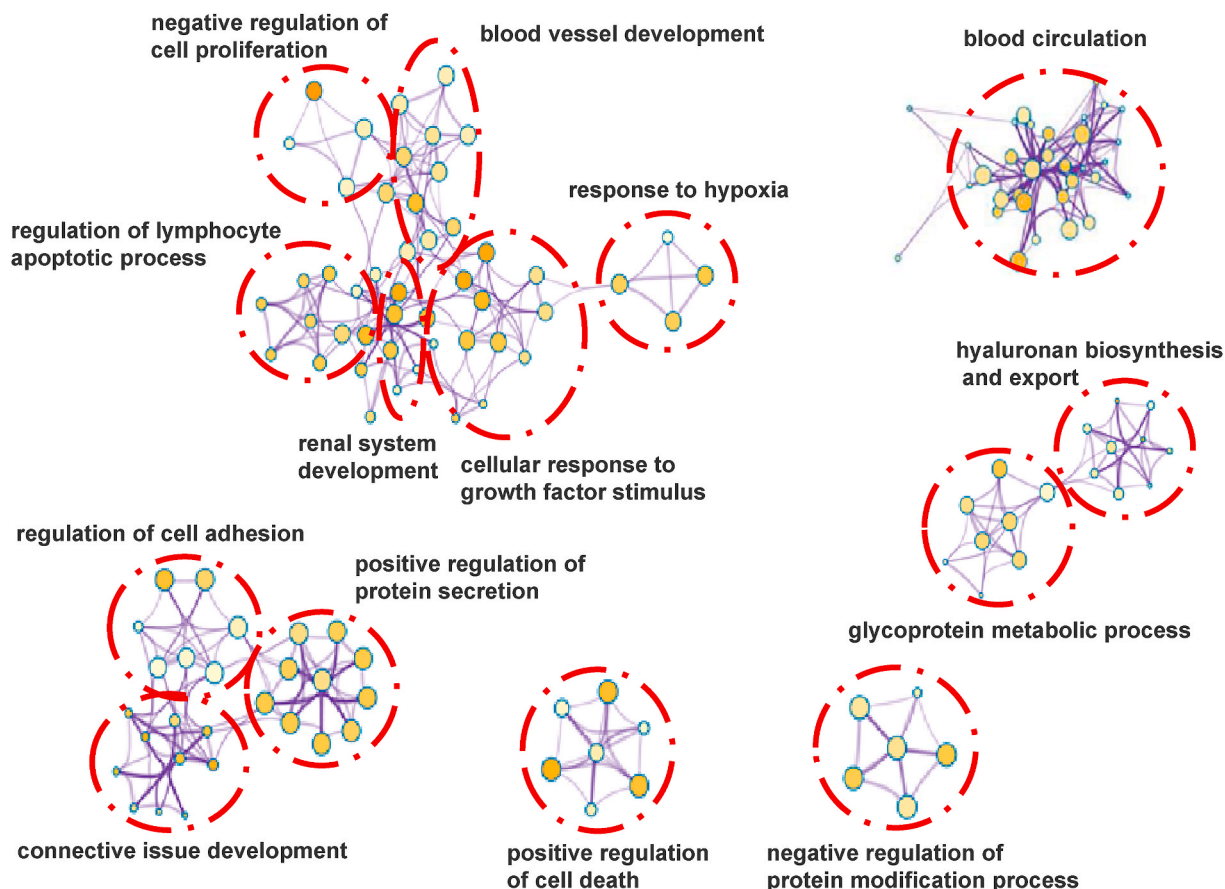


Fig. 2. Network of the enriched terms, colored by *p*-values. The terms containing more genes tended to have more significant *p*-values.

MCODE	GO	Description	Log10(P)
MCODE 1	GO:0030036	actin cytoskeleton organization	-8.2
	GO:0110053	regulation of actin filament organization	-7.8
	GO:0030029	actin filament-based process	-7.8
MCODE 2	R-HSA-500792	GPCR ligand binding	-20.3
	GO:0007188	adenylate cyclase-modulating G protein-coupled receptor signaling pathway	-19.3
	GO:0007187	G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger	-18.6
MCODE 3	R-HSA-8856828	Clathrin-mediated endocytosis	-8.7
	R-HSA-199991	Membrane Trafficking	-7.2
	R-HSA-5653656	Vesicle-mediated transport	-7.1
MCODE 4	GO:0097711	ciliary basal body-plasma membrane docking	-5.3
	R-HSA-5620912	Anchoring of the basal body to the plasma membrane	-5.3
	GO:0140056	organelle localization by membrane tethering	-4.5
MCODE 5	R-HSA-416476	G alpha (q) signaling events	-8.2
	R-HSA-373076	Class A/1 (Rhodopsin-like receptors)	-7.4
	R-HSA-500792	GPCR ligand binding	-6.9
MCODE 6	R-HSA-2562578	TRIF-mediated programmed cell death	-9.7
	R-HSA-937061	TRIF(TICAM1)-mediated TLR4 signaling	-9.6
	R-HSA-166166	MyD88-independent TLR4 cascade	-9.6

Fig. 3. The top three pathways or processes from the enrichment results for each MCODE complex.

regulation of cell proliferation, cellular response to growth factor stimulus, renal system development, regulation of lymphocyte apoptotic process, and positive regulation of cell death. The enriched REACTOME terms included Hyaluronan biosynthesis and export, and the enriched CORUM term was the BKCA-beta2AR-AKAP79 signaling complex [18] (Fig. 1). From the network plot of the enriched terms, it can be seen that several clusters were identified, including cellular response to growth factor stimulus, positive regulation of protein secretion, positive regulation of cell death, and negative regulation of protein modification process (Fig. 2). Our enrichment analysis predicted that severe damage would cause astrocyte dysfunction, leading to increased neuronal death stimulated by ACT [19]. Physiologically, ACT is present at low levels in tissues. In some pathological conditions, such as HE during ALF, the expression of ACT may be abnormally altered [20]. Because ACT is associated with several fundamental biological processes, such as inflammation, blood coagulation, and apoptosis, the results of our analysis are reasonable and demonstrate the proper reproduction of HE [21]. Our analysis also showed that the GO terms associated with the regulation of urine volume (GO: 0035809) and renal system development (GO: 0072001) were enriched after ACT treatment. ACT levels have been reported to be substantially upregulated in the urine of patients with sporadic Creutzfeldt-Jakob disease, a prion disease in which ACT accumulates in the brain [22]. Similar urinary upregulation was observed when astrocytes were treated with a combination of ACT and AVP (data not shown). Although the underlying mechanism is unclear, this observation may indicate that, when overexpressed, ACT is secreted and extensively distributed in the CNS as well as in body fluids and eventually deposits into neurons via an unknown pathway [22].

The results of REACTOME showed enrichment of Hyaluronan biosynthesis and export (Fig. 1). This result is interesting because plasma hyaluronic acid has been suggested to be a useful biomarker of later development of HE by a large cohort study [23]. Furthermore, it is known that there is a relationship between Hyaluronan and O-GlcNAcylation. Cytosolic UDP-GlcNAc is one of the substrates for hyaluronan synthesis and also a substrate of the cytosolic enzyme, O-GlcNAc transferase, which transfers GlcNAc from UDP-GlcNAc (O-GlcNAcylation) [24]. A recent study has indicated that protein O-GlcNAcylation upon ammonia-induced glucosamine synthesis triggers oxidative stress and senescence through heme oxygenase 1 (*HO1*) and NADPH oxidase 4 (*Nox4*), thereby contributing to the pathogenesis of HE [25]. Our microarray result showed that *HO1* was upregulated upon ACT treatment (FC 1.52 ± 0.03 , $n = 2$), and this effect was enhanced in the presence of AVP (FC 1.64 ± 0.04 , $n = 2$). Therefore, our results suggest that the existing of *HO1* upregulation mechanism (oxidative stress) except ammonia and endoplasmic reticulum (ER) stress [26]. However, *Nox4* was not detected under any condition. Although the reason for this result is unknown, ACT (or AVP) may interfere with *Nox4*. This possibility needs further investigation.

The results from CORUM showed enrichment of the BKCA-beta2AR-AKAP79 signaling complex [18] (Fig. 1). Astrocytes express three types of β -adrenergic receptors ($\beta1$, $\beta2$, and $\beta3$), which can elevate intracellular cAMP levels upon activation [27,28]. Therefore, our results suggest that ACT may affect the activation of these receptors. Additionally, it has been shown *in vivo* that beta2AR can interact with other Ca^{2+} pathways, including BKCa and L-type Ca^{2+} channels [29]. Therefore, it is likely that the cAMP-dependent mechanism also targets other proteins that contribute to Ca^{2+} homeostasis.

3.2. PPI network and MCODE analysis

The top three pathways or processes in the enrichment results for each MCODE complex are presented in Fig. 3. MCODE2 contains genes that are mainly related to Gs-GPCR ligand binding. These results suggest that there is a high probability that ACT acts as a ligand of Gs-GPCR.

As mentioned previously, the expression and function of AQP4 are subject to regulation by the cAMP pathway. The effect of cAMP on the

level of cellular or plasma-membrane-localized AQP4 in rat astrocytes has previously been demonstrated [30]. Furthermore, it has been shown that F-actin plays a primary role in the localization of AQP4 to the plasma membrane, and AQP4-knockdown does not compromise the ability of astrocytes to stellate in the presence of cAMP [30]. Our results indicate that MCODE1 contains processes related to the actin cytoskeleton organization, indicating they were enriched in response to ACT treatment. Similar results were found when astrocytes were treated with a combination of ACT and AVP (data not shown). However, AVP alone did not induce the enrichment of cytoskeleton-related processes (data not shown). Therefore, these results indicate that the expression and plasma membrane localization of AQP4 are induced by the upregulation of cAMP.

The Gq-signaling pathway activates the β -isoforms of phospholipase C (PLC- β), which catalyzes the hydrolysis of phosphatidylinositol biphosphate. Consequently, inositol tris-phosphate (IP3) and diacylglycerol (DAG) are produced, which are involved in intracellular Ca^{2+} release [31]. The Gq-coupled V1a AVP receptor is expressed on astrocytes [32]. Therefore, we believe that AVP treatment triggers an increase in intracellular Ca^{2+} concentration. Our results from MCODE5 showed that G alpha (q) signaling events (i.e., Gq-coupled) and GPCR ligand binding were enriched after ACT treatment, although their probabilities were not high. These results suggest that ACT may also affect Gq-GPCR activation, and ACT treatment may also increase the Ca^{2+} levels in astrocytes via this pathway. However, as ACT is a relatively large protein (54 kD), different parts of ACT may affect different GPCRs. This possibility warrants further investigation.

We have previously shown that the mRNA and protein levels of AQP4 are increased more in response to the combination of ACT and AVP than that observed upon ACT treatment alone [7], indicating a cumulative effect of ACT and AVP on the activity of AC. Similarly, our microarray results showed that the cAMP pathway gene *ADCY9* [33] was upregulated more in cells treated with the combination of ACT and AVP (FC 1.73 ± 0.04 , $n = 2$) than in those treated with ACT alone (FC 1.56 ± 0.03 , $n = 2$). Therefore, our results suggest that ACT and AVP stimulate the activity of AC.

3.3. Relationship between cAMP and Ca^{2+} in astrocytes

Ca^{2+} and cAMP have a complex relationship, which has been extensively studied in astrocytes. The second messenger cAMP has been shown to regulate the Ca^{2+} current of the voltage-dependent L-type Ca^{2+} channels [34]. Recently, epinephrine (EPI) has been shown to induce a persistent increase in cAMP levels through the activation of β -adrenergic receptors. EPI-mediated reduction in hypotonicity induces the swelling of cortical astrocytes *in vivo*, and this outcome is associated with intracellular- Ca^{2+} downregulation, which is linked to cAMP upregulation [35]. This effect may explain our result that AQP4 expression was increased when the cells were treated with a combination of ACT and AVP. Increased Ca^{2+} levels upon AVP treatment may cause additional activation of AC to downregulate Ca^{2+} , resulting in more cAMP production. However, experiments using retinal glial cells from mice have yielded contradictory results. These experiments have indicated that an increase in cAMP level may inhibit astrocyte swelling, and volume regulation is mediated by the cAMP-PKA signaling pathway [36]. Additionally, in a study on AFL rats, AQP4 expression did not directly correlate with the severity of cerebral edema or hyperammonemia [37]. Although this remains unknown, there must be some regulation there. The mechanosensitive plasma membrane cation channel transient receptor potential vanilloid 4 (TRPV-4) may be involved in the regulatory mechanism. It has been suggested that the trafficking and translocation of TRPV-4 may be mediated by cAMP because the activity of TRPV-4 is regulated by PKA [38]. It is also possible that cAMP-dependent mechanisms also target other proteins that contribute to Ca^{2+} homeostasis, such as the L-type Ca^{2+} channel [34] as mentioned above, as well as the processes that govern the translocation of AQP4 to the plasma

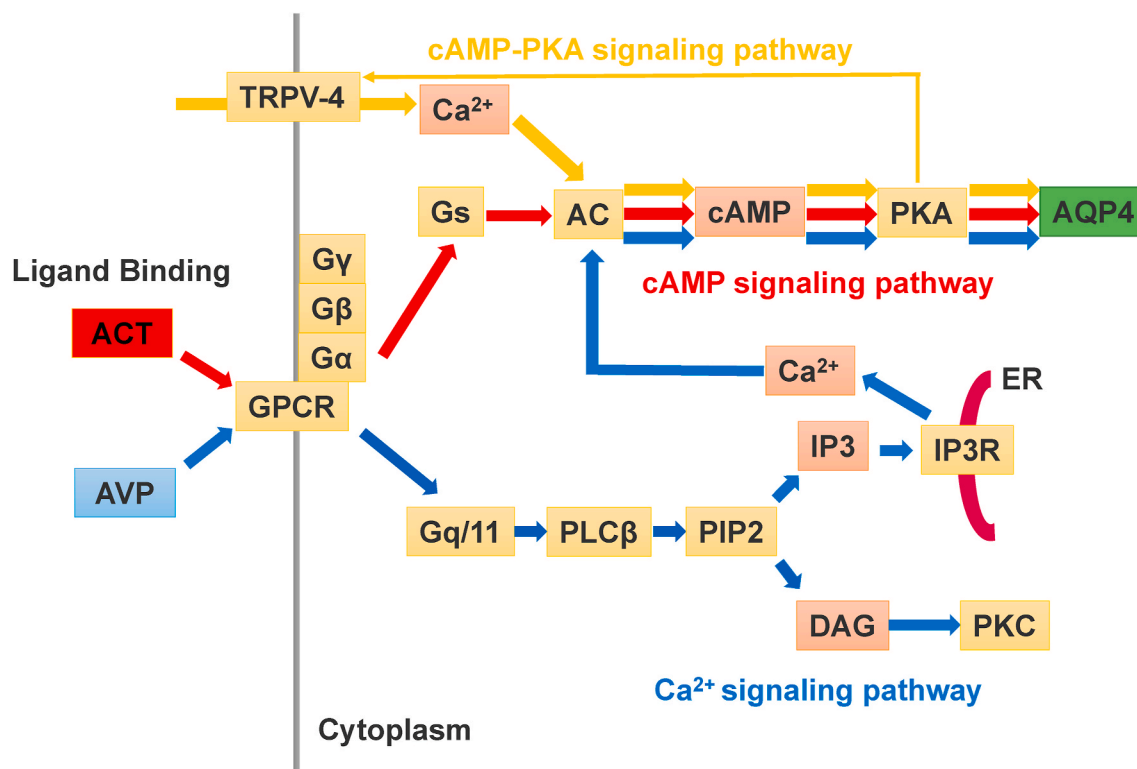


Fig. 4. The proposed pathways of how cAMP and Ca²⁺ interact and result in increased AQP4 expression via Gs and Gq/11 in the pathology of HE during ALF. In the first pathway (red arrows), Gs binds to ACT and consequently activates AC, which produces cAMP as a secondary messenger. Subsequently, cAMP activates PKA. The activated cAMP-PKA signaling pathway may increase the intake of Ca²⁺ through TRPV-4 as the second pathway (amber arrows). The Ca²⁺ intake activates AC and results in the production of more cAMP. Afterward, PKA engages in numerous reactions, including the activation of AQP4 expression. In the third pathway (blue arrows), Gq/11 binds to AVP and consequently activates phospholipase Cβ (PLCβ), which hydrolyzes the membrane phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) into two secondary messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to IP3 receptor on the ER, inducing Ca²⁺ release. The released Ca²⁺ activates AC, thereby causing AQP4 upregulation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

membrane [39], morphological changes in astrocytes [40], and phosphorylation of AQP4 [41]. In any case, at the stage of HE during ALF, the cascades may rapidly proceed toward astrocyte swelling, which means more AQP4 expression. However, further investigation is needed to elucidate these mechanisms fully.

The pathways provisionally explaining how cAMP and Ca²⁺ interact and result in AQP4 upregulation via Gs and Gq/11 in the pathology of HE during ALF are shown in Fig. 4. In the first pathway (red arrows in Fig. 4), Gs binds to ACT and thereby activates AC, which produces cAMP as a secondary messenger. Subsequently, cAMP activates PKA. The activated cAMP-PKA signaling pathway may increase the intake of Ca²⁺ through L-type Ca²⁺ channels, such as TRPV-4, as the second pathway (amber arrows in Fig. 4). The Ca²⁺ intake activates AC, consequently resulting in the production of more cAMP. Afterward, PKA participates in numerous reactions, including the activation of AQP4 expression. In the third pathway (blue arrows in Fig. 4), Gq/11 binds to AVP and consequently activates phospholipase Cβ (PLCβ), which hydrolyzes the membrane phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) into two secondary messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to IP3 receptor on the ER, inducing Ca²⁺ release. The released Ca²⁺ activates AC, resulting in AQP4 upregulation.

There is consensus among many study conclusions that ammonia, which is the main cause of HE, participates in the pathogenesis of astrocyte swelling. Some of these factors, such as glutamine, oxidative/nitrosative stress, toll-like receptor 4, and AQP4, may lead to astrocyte swelling [3]. However, there are some controversial reports on the role of AQP4 [36,37], as mentioned above. Therefore, the involvement of AQP4 in the pathology of HE cannot be explained only by hyperammonemia.

In conclusion, in cultured human astrocytes, ACT activates AC, and the presence of AVP enhances this process. Prolonged cAMP signaling can induce the expression of AQP4, thereby leading to astrocyte swelling. Although the mechanism underlying the interaction of cAMP and the intracellular Ca²⁺ signaling pathway is not completely understood, in an acute phase such as HE during ALF, the imbalance of these secondary messengers may lead to cytotoxic brain edema. Accordingly, our findings suggest that ACT may be a therapeutic target for the treatment of acute HE.

Credit author statement

Kenji Kawaguchi: Conceptualization, Data curation, Investigation, Methodology, Writing- Original draft preparation. **Jonghyuk Park:** Investigation, **Takahiro Masaki:** Investigation, Writing- Reviewing and Editing. **Yoshihiro Mezaki:** Writing- Reviewing and Editing. **Sae Ochi:** Writing- Reviewing and Editing. **Tomokazu Matsuura:** Project administration, Supervision, Writing- Reviewing and Editing.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgements

This research was supported by AMED under Grant Number JP20fk0310112.

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