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# Involvement of aquaporin in thromboxane $A_2$ receptor-mediated, $G_{12/13}$/RhoA/NHE-sensitive cell swelling in 1321N1 human astrocytoma cells

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ABSTRACT

The physiological role of the thromboxane $A_2$ ($TXA_2$) receptor expressed on glial cells remains unclear. We previously reported that 1321N1 human astrocytoma cells pretreated with dibutyryl cyclic AMP (dbcAMP) became swollen in response to U46619, a $TXA_2$ analogue. In the present study, we examined the detailed mechanisms of $TXA_2$ receptor-mediated cell swelling in 1321N1 cells. The cell swelling caused by U46619 was suppressed by expression of p115-RGS, an inhibitory peptide of $G\alpha_{12/13}$ pathway and C3 toxin, an inhibitory protein for RhoA. The swelling was also inhibited by treatment with Y27632, a Rho kinase inhibitor and 5-(ethyl-N-isopropyl)amiloride (EIPA), a $Na^+/H^+$-exchanger inhibitor. Furthermore, cell swelling was suppressed by the pretreatment with aquaporin inhibitors mercury chloride or phloretin in a concentration-dependent manner, suggesting that aquaporins are involved in U46619-induced 1321N1 cell swelling. In fact, U46619 caused $[^3H]H_2O$ influx into the cells, which was inhibited by p115-RGS, C3 toxin, EIPA, mercury chloride and phloretin. This is the first report that the $TXA_2$ receptor mediates water influx through aquaporins in astrocytoma cells via $TXA_2$ receptor-mediated activation of $G\alpha_{12/13}$, Rho A, Rho kinase and $Na^+/H^+$-exchanger.



## 1. Introduction

Thromboxane $A_2$ ($TXA_2$), an unstable arachidonic acid metabolite, plays an important role in constriction of vascular and bronchiolar smooth muscle cells, mitogenesis of vascular smooth muscle cells and platelet aggregation [1,2]. The $TXA_2$ receptor is a member of the G protein-coupled receptor (GPCR) superfamily [3], and couples with trimeric G proteins, $G_{q/11}$ and $G_{12/13}$ [2], and also with $G_{i/o}$, $G_{16}$ and $G_h$ [4–6]. This receptor is expressed in rabbit [7], rat [8], and mouse astrocytes [9] and also in human astrocytoma 1321N1 cells [10]. While $TXA_2$ receptor expression in astrocyte/astrocytoma cells exerts the secretion of interleukin-6 [9], astrogliosis [11], and surface expression of large-conductance, voltage- and $Ca^{2+}$-activated $K^+$ channels [12], the physiological role of the $TXA_2$ receptor in astrocyte/astrocytoma cells remains largely unknown.

Astrocytes have been shown to provide dynamic support for neurons by releasing neurotrophic factors. Astrocytes also play a variety of important roles in brain injury and neurodegeneration [13,14]. Pathophysiological accumulation of water in the brain, in situations such as the presence of tumors, can cause devastating neurological damage resulting in much of the morbidity and mortality in the brain [15]. Astrocytes are the site of water entry in the initial phase of brain edema formation [16]. Several studies have shown that aquaporins are important in water homeostasis and neuronal signaling of the central nervous system [17,18].

Aquaporins, a well-known water channel family of proteins, regulate water osmotic permeability on the plasma membrane [15]. There are at least 13 aquaporins in mammals [15]. Among them, the existence of aquaporin-4 in astrocytes is well known, and aquaporin-1 and -9 are also expressed in the brain [19]. Aquaporin-4 is thought to be involved in water entry in the brain [16], and in migration of astrocytes [20,21]. However, while aquaporin-4 is thought to be involved in brain edema [17,22], aquaporin-4 has also been shown to relieve vasogenic brain edema [18,23]. By contrast, although aquaporin-1 in glioma cells has

*Abbreviations:* dbcAMP, dibutyryl adenosine 3′,5′-monophosphate; EIPA, 5-(N-Ethyl-N-isopropyl)amiloride; GF109203X, (R)-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide;3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; GFP, green fluorescent protein; NHE, $Na^+/H^+$-exchanger; PD98059, 2′-amino-3′-methoxy-flavone; SQ29548, [1S-[1α,2β(5Z),3β,4α]]-7-[[3-[[2-(phenylamino)carbonyl]hydrazine]methyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid; TBST, Tris-buffered saline containing 0.05% Tween 20; $TXA_2$, thromboxane $A_2$; GPCR, G protein-coupled receptor; ROCK, Rho kinase; U466119, 9,11-dideoxy-9α,11α-epoxymethanoprostagrandin $F_{2\alpha}$; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione; Y27632, (R)-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide.

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been reported to enhance cell growth and migration [24], the physiological role of aquaporin-1 in astrocyte/astrocytoma cells remains unclear. As discussed above, the function of aquaporins in astrocytes/astrocytoma cells requires clarification.

Intracellular water volume is regulated by cation balance between intracellular and extracellular regions. One of the major cations is sodium, and activation of $Na^+/H^+$-exchanger (NHE) is involved in the movement of water to the intracellular space [25].

We previously reported that 1321N1 cells became spindle shaped in the presence of dibutyryl cyclic AMP (dbcAMP) [26], and U46619, a specific $TXA_2$ receptor agonist, caused cells swelling through $G\alpha_{12/13}$ and RhoA activation [11]. $G\alpha_{12/13}$ activates small G protein Rho and modulates the actin cytoskeleton, cytokines, cell motility and contraction [27]. $G\alpha_{12/13}$ are also known to regulate cell proliferation [28] and the activation of NHE [29]. In the present study, we investigated the involvement of aquaporins and NHE in U46619-induced cell swelling. This is the first report that U46619 caused water entry followed by cell swelling in 1321N1 cells and that this process was mediated via $G\alpha_{12/13}$, RhoA, NHE and aquaporins.

## 2. Materials and methods

### 2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Nissui Pharmaceutical, Tokyo, Japan. Dibutyryl adenosine 3′,5′-monophosphate (dbcAMP), 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) and phloretin were from Sigma Aldrich (St. Louise, MO, USA). 9,11dideoxy-9α,11α–epoxymethanoprostagrandin $F_{2\alpha}$(U466119) and [1S-[1α,2α(5Z),3α,4α]]-7-[[3-[[2-(phenylamino)carbonyl]hydrazine] methyl]-7-oxabicyclo[2,2,1]hept-2-yl]-5-heptenoic acid (SQ29548) were from Cayman Chemicals (Ann Arbor, MI, USA). 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl] amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) and (R)-(+)-trans-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632) were from CALBIOCHEM (Darmstadt, Germany). 3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (GF109203X) was from BIOMOL (Plymouth Meeting, PA, USA). 2′-amino-3′-methoxy-flavone (PD98059) and merõcuric chloride ($HgCl_2$) were from Wako Pure Chemicals (Osaka, Japan). Anti-aquaporin-1 and anti-aquaporin-9 antibodies (rabbit) were from CHEMICON (Temecula, CA, USA). HRP-conjugated anti-rabbit IgG was from Cell Signaling TECHNOLOGY (Beverly, MA, USA). $[^3H]H_2O$ was from Perkin Elmer (Boston, MA, USA).

### 2.2. Cell culture, differentiation and swelling

Human astrocytoma 1321N1 cells were cultured in a humidified atmosphere at 37 °C under 5% $CO_2$ in DMEM supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin G and 100 μg/ml streptomycin. For differentiation, 1321N1 cells were grown in DMEM containing 5% FCS for 3 days, after which cells were cultured in serum-free DMEM containing 500 μM dbcAMP for 24 h. The cells were then stimulated using 1 μM U46619 (Cayman Chemicals, Ann Arbor, MI, USA) in the presence or absence of various inhibitory peptides or compounds. After exposure to these compounds cells were fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). The cell morphology was observed under a phase-contrast microscope (IX-70, Olympus, Tokyo, Japan) [30].

### 2.3. Evaluation of cell swelling

Human astrocytoma 1321N1 cells seen in a phase-contrast photomicrograph were filled in with black using Adobe Photoshop 7.0 software. The average area of the cells was quantified by NIH image 1.61 software.

### 2.4. Preparation and infection of recombinant adenovirus

Recombinant adenovirus encoding the carboxyl terminal region of $G\alpha_q$ ($G\alpha_q$-ct; amino acids 305–359), the amino terminal region containing the RGS domain of p115-RhoGEF (p115-RGS; amino acids 1–252), and C3 toxin were prepared as described previously [31]. Each recombinant adenovirus contains a green fluorescent protein (GFP) gene, allowing direct observation of the infection state. Human astrocytoma 1321N1 cells were infected with recombinant adenovirus at a multiplicity of infection (MOI) of 100 for 1 h at 37 °C, and were then cultured for an additional 24 h. After this time cells were treated with 500 μM dbcAMP for 24 h. Under these conditions, almost 100% of cells were infected with adenovirus coding for GFP, showing green fluorescence.

### 2.5. Measurement of $[^3H]H_2O$ influx

$[^3H]H_2O$ influx was determined by the method of McConnell et al. [32] with a slight modification. Human astrocytoma 1321N1 cells were seeded onto 12-well plates coated with poly-L-lysine ($1.0\times10^5$ cells/ml). Three days after seeding, the cells were treated with several kinds of adenoviruses and 500 μM dbcAMP in the absence of FCS for 24 h. After the cells were preincubated with various inhibitors for 30 min, the cells were incubated with 1 μM U46619 in the presence of $[^3H]H_2O$ (Perkin Elmer, 1.13 μCi/ml) for 30 min. After washing the cells three times with ice cold PBS, the cells were lysed with 0.5 ml of 1 N NaOH and, following a wash with 0.5 ml of 1 N HCl, the radioactivity of $[^3H]H_2O$ in the cell lysates was determined by scintillation counting.

### 2.6. Extraction of total RNA from 1321N1 cells

Total RNA of 1321N1 cells was extracted using TriPure Isolation Kit (Roche Diagnostics) with a slight modification of the previous method [33].

### 2.7. Reverse transcription-polymerase chain reactions (RT-PCR)

RT-PCR was performed as described previously [34]. The primers for human aquaporin-1 were: sense; 5′-GGC CAC GAC CCT CTT TGT CTT CAT-3′, antisense; 5′-TCC CAC AGC CAG TGT AGT CAA TAG-3′, 514 base pairs. The primers for human aquaporin-4 were: sense; 5′-CTG GTC ATG GTC TCC TGG TT-3′, antisense; 5′-CTG TTG TCC TCC ACC TCC AT-3′, 385 base pairs. The primers for human aquaporin-9 were: sense; 5′-CGG CAT TTG TAC AGT CAG AGA CTC-3′, antisense; 5′-AAT GCG TTC GCC AGA GAT AGA TAC-3′, 632 base pairs. Each cDNA fragment was amplified 31 times by 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. PCR products, which were separated using electrophoresis on 1.5% agarose gels and stained with ethidium bromide, were analyzed by an image scanner (FAS-III, TOYOBO Co, Ltd., Osaka, Japan).

### 2.8. Western blotting

Human astrocytoma 1321N1 cells were dissolved in Laemmli sample buffer (final concentration, Tris–HCl 62.5 mM, SDS 2%, glycerol 10%, 2-mercaptoethanol 5%, pH 6.8) and incubated at 50 °C for 30 min. The electrophoresis was performed on 8% acrylamide gels. Proteins were transferred electrically from the gel onto polyvinylidene difluoride membrane by the semi-dry blotting method. The blots were blocked for 1 h with 2% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST: 10 mM Tris–HCl, 100 mM NaCl, and 0.05% Tween 20, pH 7.5) at room temperature, and incubated with an antibody to aquaporin-1 or aquaporin-9 at 1000 times dilution in TBST containing 2% non-fat dry milk at 4 °C for overnight. The blots were washed with TBST and incubated with a 10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody in TBST containing 1% non-fat dry milk at