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Role of actin in the cAMP-dependent activation of sodium/glucose cotransporter in renal epithelial cells

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Abstract

We examined whether actin filaments are involved in the cAMP-dependent activation of a high affinity sodium/glucose cotransporter (SGLT1) using epithelial expression systems. The expression of enhanced green fluorescent protein-tagged SGLT1 (EGFP-SGLT1) in Madin–Darby canine kidney (MDCK) cells was revealed by Western blotting and confocal laser microscopy. 8-Br-cAMP, a membrane permeable cAMP analog, enhanced [^{14}C]-α-methyl glucopyranoside ([^{14}C]-AMG) uptake. Both basal and 8-Br-cAMP-elicited [^{14}C]-AMG uptakes were inhibited by *N*-(2{[3-(4-bromophenyl)-2-propenyl]-amino}-ethyl)-5-isoquinolinesulfonamide (H-89), a protein kinase A inhibitor, and cytochalasin D, an actin filament formation inhibitor. Furthermore, cytochalasin D inhibited the distribution of EGFP-SGLT1 at the apical surface. These results suggest that the EGFP-SGLT1 protein is functionally expressed in the apical membrane of MDCK cells, and is up-regulated by a cAMP-dependent pathway requiring intact actin filaments.

Keywords: Green fluorescent protein; Protein kinase A; SGLT1

1. Introduction

The transepithelial transport of glucose from the lumen to the serosa requires two different processes in epithelial cells. Sodium-dependent glucose transport occurs in the apical membrane, and the facilitated diffusion of glucose occurs in the basolateral membrane. The former is involved in the absorption and reabsorption of glucose in the intestine and kidney, respectively. So far, four types of sodium/glucose cotransporter, SGLT1, SGLT2, SGLT3, and NaGLT1, have been cloned, of which SGLT1 has been well characterized [1–3]. Furthermore, exogenous expression studies of SGLT1 have been performed using epithelial cells [4,5], *Xenopus* oocytes [5,6] and COS-7 cells [7].

Protein kinases alter the function of membrane transport proteins. The elevation of cAMP increased the cell surface expressions of Na^+/K^+-ATPase [8], cystic fibrosis transmembrane conductance regulator Cl^- channel [9] and Na^+-taurocholate cotransporting peptide (Ntcp) [10]. Similarly, the activation of protein kinase A (PKA) increased the maximum rate of sodium/glucose cotransport in SGLT1-expressing *Xenopus* oocytes [11]. The cAMP-dependent vesicle trafficking may regulate SGLT1 exocytosis and insertion into the apical membrane. However, these mechanisms have not been examined in epithelial expression systems.

In the present study, we established Madin–Darby canine kidney (MDCK) cells expressing enhanced green fluorescent protein-tagged SGLT1 (EGFP-SGLT1), which facilitated the immunodetection of SGLT1 with an anti-EGFP antibody and by direct fluorescence detection. We found that the apical surface expression of SGLT1 is regulated by a cAMP-dependent pathway requiring intact actin filaments.

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2. Materials and methods

2.1. Plasmid cDNA constructs

The translated region of SGLT1 cDNA was cloned from the kidney of a Wistar male rat by the reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA was subcloned into the mammalian pEGFP-N1 vector (Clontech, CA, USA) to obtain an EGFP-SGLT1 vector.

2.2. Cell culture and transfection

MDCK cells were obtained from the European Collection of Cell Cultures (#84121903, Wiltshire, UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma) in an atmosphere of 5% CO_2 at 37 °C. Subculturing was done every 3–4 days using 0.02% EDTA and 0.25% trypsin. For the transfection procedure, cells were seeded into 35 mm plastic dishes and transfected with the EGFP-SGLT1 vector the next day by SuperFect Reagent (QIAGEN, CA, USA). Two days after transfection, the cells were exposed to a medium containing 0.4 mg/ml G418 sulfate and maintained in the continuous presence of the selecting drug.

2.3. SDS-polyacrylamide gel electrophoresis and Western blotting

Cell lines expressing the EGFP-SGLT1 protein were screened by Western blotting. The total and apical membrane fractions were prepared as described by Turner and Moran [12]. The protein concentration was measured using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was carried out as previously described [13]. In brief, membrane preparations (40 μg) were applied to the SDS-polyacrylamide (10%) gel. Proteins were blotted onto a PVDF membrane and then the membrane was incubated with a primary antibody (anti-green fluorescent protein antibody, Rockland Immunochemicals, PA, USA) and a secondary antibody (peroxidase conjugated anti-goat IgG). Finally, the blots were stained with ECL Western Blotting Detection Reagents (Amersham Pharmacia Biosciences, Buckinghamshire, UK).

2.4. Immunofluorescence microscopy

The cells were seeded on coverslips in 6-well plates. After fixation with 3% paraformaldehyde for 7 min at room temperature, the coverslips were mounted using Vectashield (Vector Laboratories, CA, USA). The expression of EGFP-SGLT1 was visualized on an LSM 510 confocal microscope (Carl Zeiss, Germany) set up with a filter for green fluorescence detection (488 nm excitation, 509 nm emission filter). Images were collected at 1.0-μm increments (vertical direction) beginning at the basal membrane and ending at the apical membrane. Images were further processed using the Adobe Photoshop (Adobe System, San Jose, CA, USA).

2.5. α-Methyl glucopyranoside (AMG) uptake

Cells were grown to confluent densities on 24-well plates. [^{14}C]-AMG uptake was assayed by incubating in Hank's balanced salt solution (HBSS) containing 0.1 mM unlabeled AMG, 0.4 μCi/ml [^{14}C]-AMG (Perkin Elmer Life Sciences, Boston, MA, USA), 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.4). After incubation for the indicated period at 37 °C, the solution was aspirated quickly and washed by ice-cold HBSS without [^{14}C]-AMG four times. The cells were solubilized with 0.5 N NaOH and aliquots were taken to determine the radioactivity and protein concentration.

2.6. Statistics

Results are presented as means±S.E. Differences between groups were analyzed by one-way ANOVA, and corrections for multiple comparisons were made by Tukey's multiple comparison test. Comparisons between the two groups were made by Student's t test. Significant differences were assumed at $P<0.05$.

3. Results and discussion

Rat SGLT1 cDNA was inserted at the 5′ side of the EGFP cDNA. This construct encoded a 251 amino acid sequence in addition to the original 665 residues found in rat SGLT1. The molecular size of the EGFP-SGLT1 protein was presumed to be about 100 kDa. We ascertained by RT-PCR that the renal tubule epithelial cell line, MDCK, does not express SGLT1 endogenously (data not shown). We first generated cell lines overexpressing the EGFP-SGLT1 protein. In Western blotting analysis, a specific band for EGFP-SGLT1 appeared in the stable cell line, but did not in non-transfected cells (Fig. 1A). Confocal laser microscopy showed that EGFP-SGLT1 was distributed in the apical surface of MDCK cells (Fig. 1B). Next, the function of EGFP-SGLT1 was assessed by [^{14}C]-AMG uptake. In MDCK cells expressing EGFP-SGLT1, [^{14}C]-AMG uptake was about 20 times higher than that of non-transfected cells (Fig. 2A). This [^{14}C]-AMG uptake was almost completely inhibited by phloridzin (500 μM), a potent inhibitor of SGLT1 (the inhibitory percentage was 97.6±0.2%). The [^{14}C]-AMG uptake increased time-dependently and reached a maximum over 120 min (Fig. 2B). The initial rate of [^{14}C]-AMG uptake was estimated by measuring its accumulation at 15 min over a range of substrate concentrations (0.02 to 1 mM). The K_m value for AMG was 376 μM. This value is similar to those of rat SGLT1 expressed in *Xenopus* oocytes (397 μM) [13] and myc-tagged human renal SGLT1 expressed in *Xenopus*

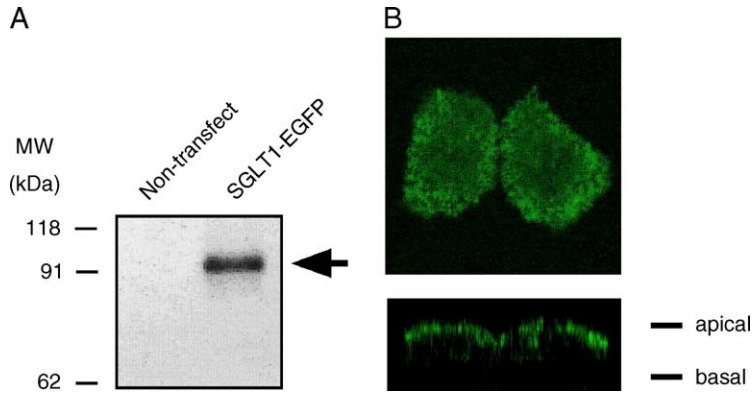

Fig. 1. Expression of EGFP-SGLT1 in MDCK cells. (A) Total membrane fractions (30 μg) were prepared from non-transfected and EGFP-SGLT1 transfected cells. Proteins were detected with an anti-EGFP polyclonal antibody. Molecular weights are indicated on the left side. The specific band of EGFP-SGLT1 (100 kDa) is indicated by an arrow. (B) Images from confocal laser microscopy show the localization of EGFP-SGLT1. The upper image shows *x–y* apical side scans. The lower image shows *x–z* scans performed in 1.0-μm steps over a range of 10 μm.

oocytes (0.56 mM) [5], indicating that glucose binding and/or transport are not affected by tagging with EGFP. There are some reports on the functional expression of epitope-tagged SGLT1 protein [4,5,7,14], indicating that epitope tags might interfere with the synthesis, folding, trafficking, and function of membrane proteins. Turner et al. [4] reported that carboxyl-terminal vesicular stomatitis virus G (VSV-G) protein-tagged rabbit SGLT1 was functionally expressed in Caco-2 cells. However, the VSV-G-SGLT1 was mistargeted to the basolateral membrane, possibly due to the deletion of carboxyl-terminal bases 1996–2010 of SGLT1 and/or the addition of the basolateral targeting sequence of VSV-G. In the present study, we found that carboxyl-terminal EGFP-tagged rat SGLT1 was functionally expressed and targeted to the apical membrane in MDCK cells according to the specific sorting sequence of SGLT1 [15].

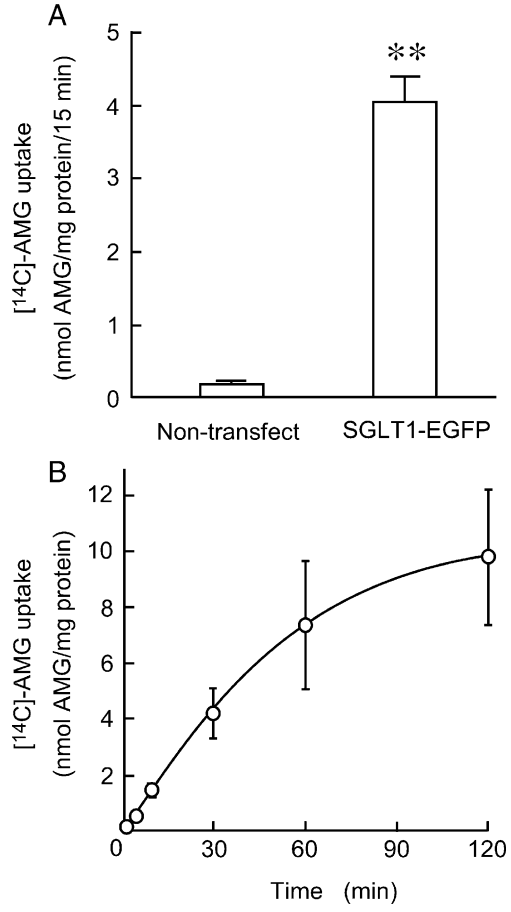

Fig. 2. Transport characteristics of EGFP-SGLT1. (A) [^{14}C]-AMG uptake was carried out at 37 °C for 15 min in non-transfected and EGFP-SGLT1-expressing MDCK cells. ** P<0.01, significantly different from the value in non-transfected cells. (B) [^{14}C]-AMG was incubated for indicated periods in EGFP-SGLT1-expressing MDCK cells.

In the *Xenopus* oocytes expression system, Hirsch et al. [11] reported that 8-Br-cAMP, a membrane permeable cAMP analog, increases the maximum rate of sodium/glucose cotransport and the plasma membrane area, indicating that cAMP/PKA increases the trafficking of rabbit and human SGLT1 to the plasma membrane. Furthermore, in isolated membranes from the rat small intestine [16] and rat jejunum [17], glucose transport and the apical expression of SGLT1 are upregulated by PKA. However, the effects of cAMP/PKA on SGLT1 trafficking have not been examined using epithelial cells. We found that 8-Br-cAMP (250 μM) enhanced [^{14}C]-AMG uptake in MDCK cells expressing EGFP-SGLT1 (Fig. 3A). Both basal and cAMP-elicited [^{14}C]-AMG uptakes were significantly inhibited by 50 μM *N*-(2{[3-(4-bromophenyl)-2-propenyl]-amino}-ethyl)-5-isoquinolinesulfonamide (H-89), a PKA inhibitor [18]. We recently reported that cAMP/PKA up-regulates the trafficking of SGLT1 mediated by phosphatidylinositol 3-kinase activation in LLC-PK$_1$ cells [19]. However, the trafficking mechanisms of SGLT1 have not been clarified.

The trafficking of ion transporters from the intracellular compartment to the plasma membrane is a key step in the development of ion transport function. If the effects of cAMP in the trafficking of EGFP-SGLT1 to the cell surface depend on microfilament-based motility mechanisms, the disruption of actin filaments would be expected to affect the trafficking of EGFP-SGLT1. Cytochalasin D (10 μM), an

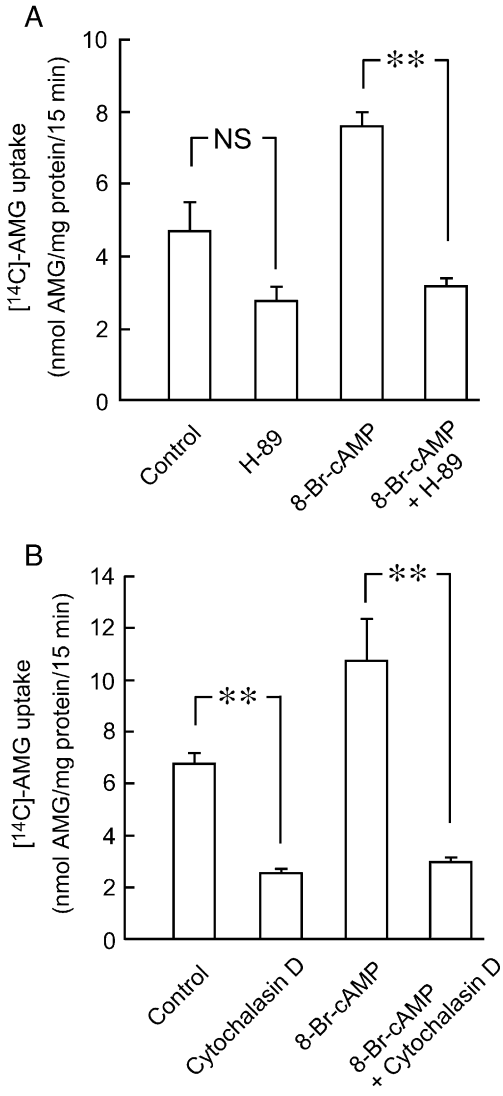

Fig. 3. Effects of cytochalasin D on 8-Br-cAMP-activated AMG uptake. (A) The cells were pre-incubated with H-89 (50 μM), 8-Br-cAMP (250 μM), or both of them for 2 h, followed by incubation with [^{14}C]-AMG for 15 min. (B) The cells were pre-incubated with cytochalasin D (10 μM), 8-Br-cAMP (250 μM), or both of them for 2 h, then incubated with [^{14}C]-AMG for 15 min. **P<0.01.

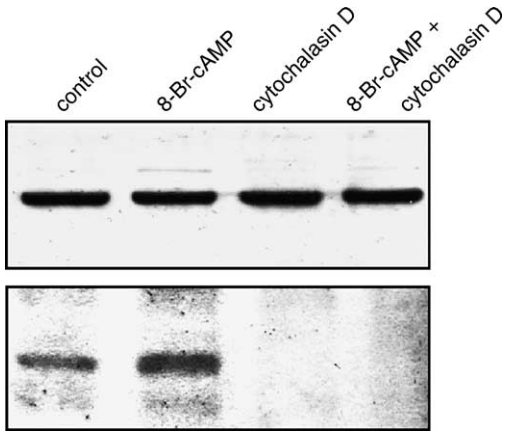

Fig. 5. Effect of cytochalasin D on the apical surface distribution of EGFP-SGLT1. The cells were pre-incubated with 8-Br-cAMP (250 μM), cytochalasin D (10 μM), or both of them for 2 h. The total (upper) and apical (lower) membrane fractions were run on SDS-polyacrylamide gels and immunoblotted with an anti-EGFP antibody.

inhibitor of actin polymerization [20], significantly inhibited both basal and cAMP-elicited [^{14}C]-AMG uptakes (Fig. 3B). Furthermore, the apical surface localization of EGFP-SGLT1 was inhibited by cytochalasin D in the presence and absence of 8-Br-cAMP (Fig. 4). Similarly, Western blotting showed that cytochalasin D inhibited the apical distribution of EGFP-SGLT1 without changing protein expression in the total membrane fraction (Fig. 5). These results indicate that intact actin filaments are necessary to translocate SGLT1 from cytosolic compartments to the plasma membrane. Chung et al. [21] reported that cytochalasin D inhibited epidermal growth factor-elicited glucose transport and SGLT1 expression in the brush border membrane using a rabbit in vivo model. Our in vitro experiments are consistent with their results.

In conclusion, we have established a stable expression system for EGFP-SGLT1 in MDCK cells. Our results suggest that the activation of the cAMP/PKA pathway increases glucose transport mediated by the trafficking of EGFP-SGLT1 to the apical surface. This trafficking system requires intact actin filaments. Missense mutations in the

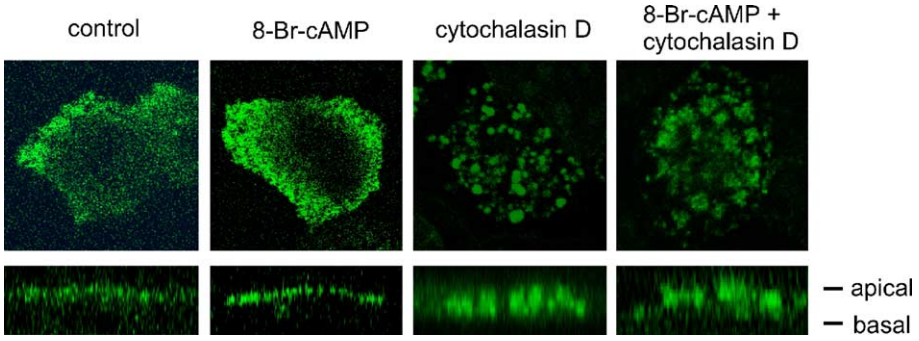

Fig. 4. Effect of cytochalasin D on EGFP-SGLT1 localization. Images from confocal laser microscopy showed the intracellular localization of EGFP-SGLT1. The cells were pre-incubated with 8-Br-cAMP (250 μM), cytochalasin D (10 μM), or both of them for 2 h. The upper image shows x–y apical side scans. The lower image shows the x–z scans performed in 1.0-μm steps over a range of 10 μm.

trafficking defects of SGLT1 have been reported in glucose–galactose malabsorption [22]. This cell line-expressing EGFP-SGLT1 will be useful to clarify trafficking mechanisms in a future study.

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