



Phloridzin inhibits high K⁺-induced contraction via the inhibition of sodium: glucose cotransporter 1 in rat ileum

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ABSTRACT. Recent studies have shown that phloridzin, an inhibitor of sodium–glucose cotransporter (SGLT), strongly decreases high K⁺-induced contraction in phasic muscle, such as tenia coli, but slightly affects tonic muscle, such as trachea. In this study, we examined the inhibitory mechanism of phloridzin on high K⁺-induced muscle contraction in rat ileum, a phasic muscle. Phloridzin inhibited the high K⁺-induced contraction in the ileum and the aorta, and the relaxing effect of phloridzin at 1 mM in the ileum was approximately five-fold more potent than that in the aorta. The expression of SGLT1 mRNA in the ileum was higher than that of the aorta. Phloridzin significantly inhibited NADH/NAD ratio and phosphocreatine (PCr) content in the ileum; however, application of pyruvate recovered the inhibition of contraction and PCr content, but had no effect on ratio of NADH/NAD. High K⁺ increased 2-(N (7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) uptake in ileal smooth muscle cells, and phloridzin inhibited the increase in a concentration-dependent manner. These results suggest that phloridzin inhibits high K⁺-induced contraction because of the inhibition of energy metabolism via the inhibition of SGLT1.

KEY WORDS: glucose uptake, ileum, phloridzin, SGLT1, smooth muscle

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Glucose is one of the most important energy substrates for the maintenance of cell activities. Glucose uptake into cells is achieved by glucose transporter (GLUT) and/or sodium–glucose cotransporter (SGLT). SGLT 1 and 2 are known for transporting glucose with sodium. SGLT1 exists in the kidney, heart and skeletal muscle, and SGLT2 exists in the kidney [33]. However, there is no report describing the distribution of SGLTs in smooth muscle.

On the basis of their electrophysiological and mechanical behaviors, smooth muscles are classified as either phasic or tonic muscles [9, 16, 27]. Phasic smooth muscles are electrically quiescent in the resting condition and reveal spontaneous electrical spikes. Depolarization with high K⁺ induces their initial phasic contraction, which is then followed by a decline to a low steady-state level of spike activity [8]. Phasic muscles include the ileum, urinary bladder, uterus and vas deferens. In contrast, tonic smooth muscles show depolarization with or without low-level continuous spike activities. Their high K⁺-induced depolarization typically evokes a slowly developing sustained contraction as found in tonic muscles, such as the aorta and trachea. The contractile differences among different smooth muscles appear to be attributed to various pathways found in oxidative metabolism [4, 10], as well as differences in the protein expression [1, 5, 18, 21, 30] and electrophysiological responses [3, 32].

Application of hyperosmotically added high K⁺ solution induces a sustained contraction, whereas an isosmotically substituted high K⁺, Na⁺-deficient solution induces contraction followed by gradual relaxation in various smooth muscles. The possible mechanisms of relaxation are classified in three types: (1) swelling of the cells as in rabbit aorta [29], guinea pig trachea [26] and bovine trachea [12]; (2) inhibition of glucose uptake as in guinea pig urinary bladder [26] and guinea pig ileum [25]; and (3) the combination of swelling of cells and inhibition of glucose uptake as in guinea pig gallbladder [26], guinea pig tenia coli [6, 31] and porcine trachea [13]. Possible inhibition of glucose uptake in some smooth muscles by isosmotic high K⁺, Na⁺-deficient solution is

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Table 1. Gene-specific primers for real time RT-PCR

| Primer | Nucleotide sequence 5'→3' | Product size | Accession |
|---------|---------------------------|--------------|-----------|
| GAPDH F | ATGGTGAAGGTCGGTGTGAA | | |
| GAPDH R | AATGAAGGGGTCGTTGATGG | 108 | NM_017008 |
| SGLT1 F | TACATCAAGGCTGGGGTGGT | | |
| SGLT1 R | AGCAGGGACAGAACGGAAAG | 95 | NM_013033 |
| SGLT2 F | TGTGGGATGAGCAAGAGTGG | | |
| SGLT2 R | ATGAGCAGGGCATTGAGGTT | 134 | NM_022590 |

due to low Na⁺ concentration, thereby probably inhibiting SGLT.

Phloridzin, an inhibitor of SGLT, decreases high K⁺-induced contraction, in guinea pig tenia coli, a phasic muscle [28], but not in bovine trachea, a tonic muscle [13]. Moreover, phloridzin does not affect the contraction induced by high K⁺ solution containing pyruvate instead of glucose physiological salt solution (PSS) in guinea pig tenia coli [28]. However, some researchers reported that a high concentration of phloridzin (more than 10⁻⁴ M) inhibited aerobic metabolism and caused mitochondrial swelling [17]. Moreover, GLUT4 is very important to maintain muscle contraction in mouse aorta, a tonic muscle [23]. Therefore, the relationship between relaxation and inhibition of glucose uptake by phloridzin in phasic smooth muscle is not clear.

The aim of this study was to examine the relaxing mechanisms of phloridzin in rat ileum. To determine the mechanisms, high K⁺-induced muscle contraction and expression of SGLT mRNA in the ileum (phasic muscle) were compared with those in the aorta (tonic muscle), and activity of the glycolysis system, concentration of phosphocreatine (PCr) and ATP, and glucose uptake were measured.

MATERIALS AND METHODS

Animals

Male Wistar rats (250–300 g) were anesthetized using sodium pentobarbital (50 mg/kg, i.p.) and euthanized by exsanguination. The ileum, aorta and kidney were quickly removed from each animal. Eyes and renal artery from adult pigs of either sex were obtained from a local abattoir. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals at the Veterinary and Life Science University.

Muscle strip preparation and tension measurement

The longitudinal muscle layer was stripped from the ileum circular muscle as described by Paton and Aboo Zar [24]. The thoracic aorta was cut into spiral strips, and the endothelium was removed by gentle rubbing with absorbent cotton. The longitudinal muscle, which was divided into strips approximately 5–6 mm in width and 15 mm in length, and the thoracic aorta and renal artery strips of approximately 2–3 mm in width and 8–10 mm in length, two strips of iris sphincter muscles were cut from each eye (with the ciliary margin removed), were incubated with PSS containing (in mM) 136.8 NaCl, 5.4 KCl, 2.5 CaCl₂, 1.0 MgCl₂, 11.9 NaHCO₃ and 5.5 glucose. The PSS was aerated with 95% O₂ and 5% CO₂ to adjust to pH 7.2 and maintained at 37°C. For inducing hypoxia, PSS was aerated with 95% N₂ instead of O₂.

Muscle tension was isometrically recorded. One end of each strip was bound to a glass holder, and the other end was connected by a silk thread to a strain gauge transducer (TB-611T; Nihon Kohden, Tokyo, Japan) in an organ bath containing PSS with a resting tension of 0.5 g. The muscle strips were equilibrated for 30 min to obtain stable contractility induced by hyperosmotic 65 mM KCl (H-65K⁺).

Real-time RT-PCR analysis

Total RNA was subsequently extracted from the kidney, aorta (removing adventitial layer and endothelium) and ileal longitudinal muscle with TRIzol (Invitrogen Japan, Tokyo, Japan), and then, obtained RNA was suspended to a concentration of 1 µg/µl in RNase-free distilled water. Real-time RT-PCR was performed to evaluate the expression of SGLT1 and SGLT2 mRNA. The concentration of isolated RNA was measured by spectrophotometer and adjusted to 10 ng/µl. The gene expression levels were measured by real-time PCR. The PCR samples were amplified at 40 cycles at 95°C for 10 sec, 61°C for 34 sec and 42°C for 5 min. Gene-specific primers are shown in Table 1. After the amplification, mRNA was detected by PRISM 7000/7700 Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.). The relative quantification in gene expression was determined using the 2^{-ΔΔCt} method [19]. Using this method, we obtained the fold changes in gene expression normalized to an internal control gene. GAPDH was used as internal control to normalize.

NADH/NAD analysis

Approximately 10 mg of longitudinal muscle was removed, and reagents were added to create the same conditions of the measurement of muscle tension. Each sample was composed of four muscle strips. After that, the samples were rapidly frozen in liquid nitrogen and stored at -80°C until homogenized. After homogenization, the samples were added to 50 µl of 0.25 M sucrose and centrifuged at 600×g for 10 min to precipitate nuclei out of the sample. Then, samples were added to 50 µl of 0.25 M

sucrose and centrifuged at $8,000 \times g$ for 10 min to precipitate mitochondria out of the sample. NADH and NAD of samples were measured by Fluorescent NAD/NADH Detection Kit (Cell Technology, Fremont, CA, U.S.A.). NADH and NAD were detected by measurement of 590–600 nm wavelength fluorescence excited by 530–570 nm wavelength. This analysis was conducted in a low-temperature room at 4°C or on ice to prevent loss of NADH. Values of NADH and NAD were designated as a ratio of NAD to NADH.

Assay of PCr and ATP

PCr and ATP contents in the muscle strips were measured using HPLC as reported previously [14]. Muscle strips were incubated with PSS for 30 min and contracted by H-65K^{+} . After the contraction, all reagents were added to the muscle strips for 20 min. After that, the muscles were rapidly frozen in liquid nitrogen and stored at -80°C until homogenized in 6% perchloric acid (0.9 ml). The homogenate was centrifuged at $15,000 \times g$ for 5 min, and the supernatant was neutralized with 0.2 ml of 2M KHCO_3 . The neutralized extracts were spun once more, and 20 μl of supernatant was applied to the HPLC.

The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a pump (LC-10AT), a system controller (SCL-10AT), an auto injector (SIL-10AF), a column oven (CTO-10A) and a wave length-selectable detector (SPD-10Ai) set at 216 nm.

Chromatography was performed by $\mu\text{RPC C2/C18 ST}$ (4.6 mm internal diameter and 100 mm length, GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire, U.K.) using mobile phases of 50 mM KH_2PO_4 and 5 mM terabutylammonium hydrogen sulfate (TBAHS) (pH 6.0, buffer A), and 50 mM TBAHS and 40% methanol (pH 6.0, buffer B). Flow rate was 1.0 ml/min, and the elution was started with 65% buffer A. In the first 14 min, buffer B increased at a rate of 2.5%/min. This was followed by elution with 70% buffer B for 20 min and then with 100% buffer A for 10 min. These procedures were programmed with a system controller. The sensitivity of detector was usually set at 1.0 absorbance units full scale and the oven temperature at 40°C . PCr and ATP contents are expressed as $\mu\text{mol/g}$ wet weight.

Isolation of ileal smooth muscle cells

Isolation of ileal smooth muscle cells was performed by modification of Okamoto's method [22]. In briefly, the removed ileum was treated with collagenase (0.2 mg/ml) (Wako Pure Chemical, Osaka, Japan) and papaine (0.2 mg/ml) (Wako) for 1 hr to isolate smooth muscle cells. Isolated cells were prepared to adjust about 1.0×10^6 (cells/ml) with D-MEM (Wako Pure Chemical). Prepared cells were plated in amounts of 300 μl onto a cover glass in microplate and incubated for 1 hr at 37°C in D-MEM.

Assay of 2-NBDG fluorescence

Incubated cells on cover glasses were used for each uptake experiment. After incubation, medium was changed to No-glucose D-MEM (Wako) containing 1 mM 2-(N (7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) (Cayman Chemical, Ann Arbor, MI, U.S.A.), hoechst33342 (1:500) (Wako) and 65 mM KCl. Phloridzin was added at the same time, in concentrations of 10 and 100 μM . After 20 min, buffer was removed and rinsed three times with D-PBS buffer (Wako). Cells were incubated with 4% paraformaldehyde for 30 min and 2% BSA for 20 min in D-PBS buffer at 4°C . After incubation, cells were incubated with alpha-smooth muscle actin ($\alpha\text{-SMA}$) (Sigma-Aldrich, St. Louis, MO, U.S.A.) overnight. Incubated cells were rinsed with D-PBS, and the cover glass was put onto a glass slide for examination using an Axiovert 200 M fluorescence microscope (Carl Zeiss Japan, Tokyo, Japan). The excitation and emission wavelength to detect 2-NBDG were 488 nm and 560 nm.

2-NBDG, hoechst33342 and $\alpha\text{-SMA}$ fluorescence were detected by the Axiovert 200 M. Hoechst and $\alpha\text{-SMA}$ double-positive cells were determined in smooth muscle cells. Assay of 2-NBDG uptake was determined by measuring the fluorescent intensity using image J software.

Statistics

Values are expressed as mean \pm S.E.M. Statistical analyses were performed by Student's *t*-test. $P < 0.05$ or $P < 0.01$ was considered significant.

RESULTS

Effect of phloridzin on high K^{+} -induced contraction in rat ileum and aorta

An application of hyperosmotically added 65 mM KCl (H-65K^{+})- induced phasic contraction followed by sustained contraction in rat ileum and porcine iris sphincter (Fig. 1A and 1C). On the other hand, H-65K^{+} - induced only sustained contraction in rat aorta and porcine renal artery (Fig. 1B and 1D). H-65K^{+} -induced contraction became stable in 20 min after application of H-65K^{+} . Phloridzin inhibited H-65K^{+} -induced contraction in a concentration-dependent manner in rat ileum ($18.0 \pm 0.2\%$ at 300 μM and $67.9 \pm 1.1\%$ at 1 mM). Moreover, 1 mM phloridzin inhibited H-65K^{+} -induced contraction $57.6 \pm 6.1\%$ in porcine iris sphincter. On the other hand, 1 mM phloridzin slightly inhibited H-65K^{+} -induced contraction in rat aorta ($13.3 \pm 3.6\%$) and porcine renal artery ($1.6 \pm 0.7\%$) (Fig. 1E).

Expression of SGLT1 and SGLT2 mRNAs in rat ileum and aorta

SGLT1 and SGLT2 mRNAs expressions were compared between rat ileum and aorta by using real-time PCR. SGLT1 and SGLT2 mRNAs in kidney were used for a positive control. SGLT1 mRNA was significantly more highly expressed in the ileum than the aorta (Fig. 2A). However, SGLT2 mRNA expression of the ileum was similar to that of the aorta (Fig. 2B).

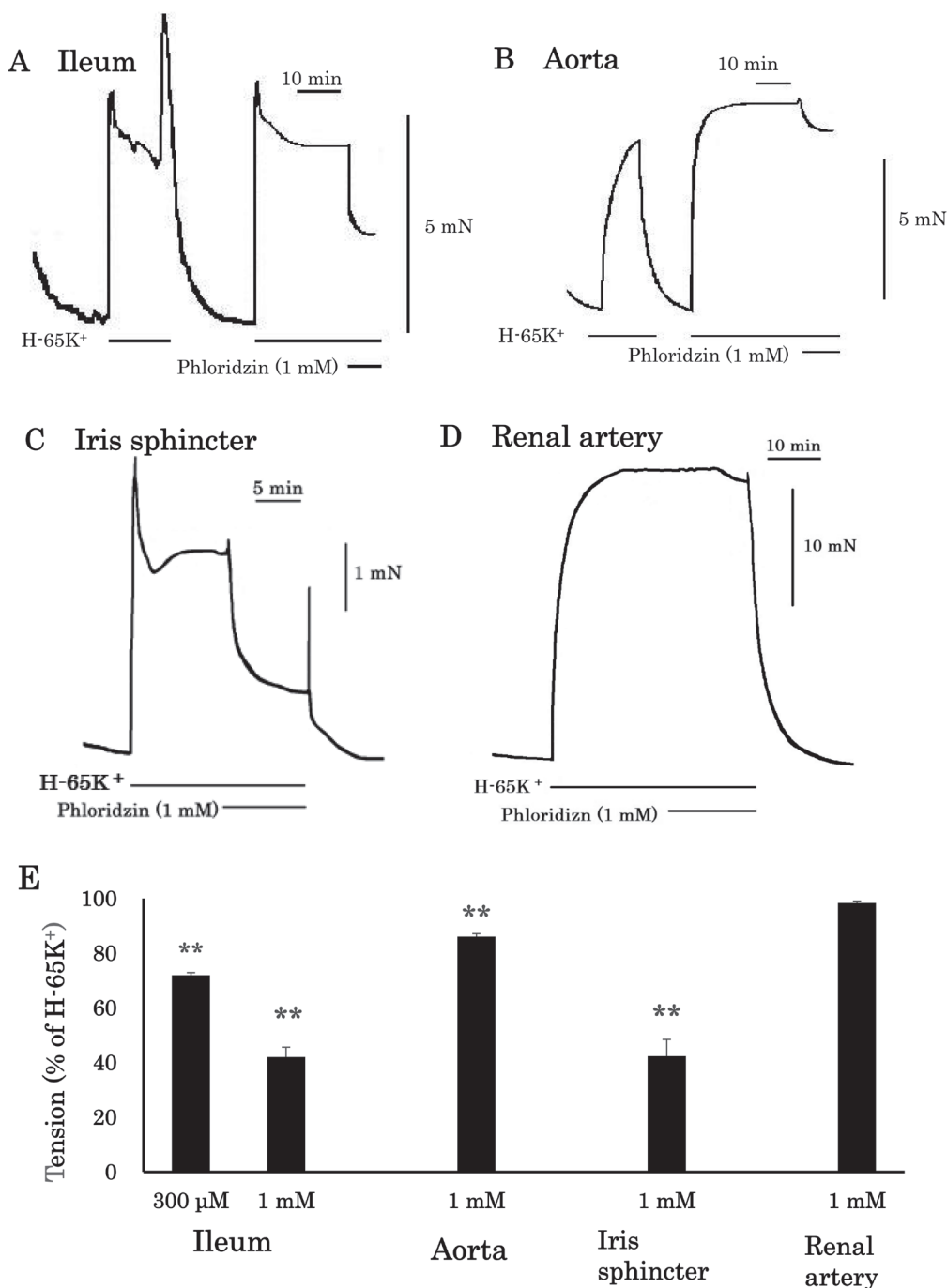


Fig. 1. Effect of phloridzin on H-65K⁺-induced contraction rat ileum and aorta. The typical trace of H-65K⁺-induced muscle contraction and phloridzin-induced relaxation in rat ileum (A), aorta (B), iris sphincter (C) and renal artery (D). E; Values of phloridzin-induced relaxation. All values were obtained 15 min after application of phloridzin. Tension of just before application of H-65K⁺ was assigned as 100%. The data are expressed as means ± S.E.M. (N=4). **Significantly different from the vehicle column ($P < 0.01$).

Effect of phloridzin on activity of glycolytic system in rat ileum

In the presence of H-65K⁺, aeration with N₂ instead of O₂ significantly increased the ratio of NADH/NAD (Fig. 3). It is known that exposure to hypoxia increases the activity of the glycolytic system in smooth muscles [20]. Thus, this result suggests that the ratio of NADH/NAD is related to the changes in the activity of the glycolytic system. Phloridzin (300 μM and 1 mM) significantly inhibited the ratio of NADH/NAD (Fig. 3). Moreover, additional application of 5.5 mM pyruvate did not recover the phloridzin-induced inhibition of NADH/NAD (Fig. 3).

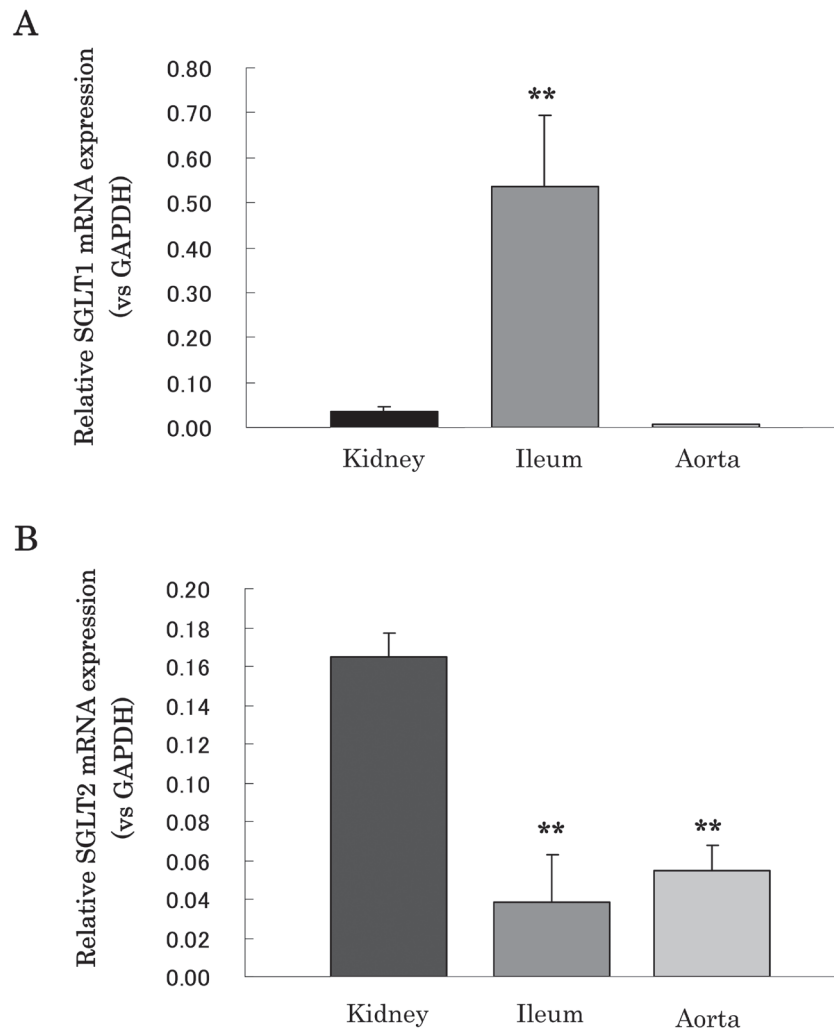


Fig. 2. Expression of SGLT1 (A) and SGLT2 (B) mRNAs in rat ileum, aorta and kidney. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize. The data are expressed as means \pm S.E.M. (N=4). **Significantly different from the kidney column ($P<0.01$).

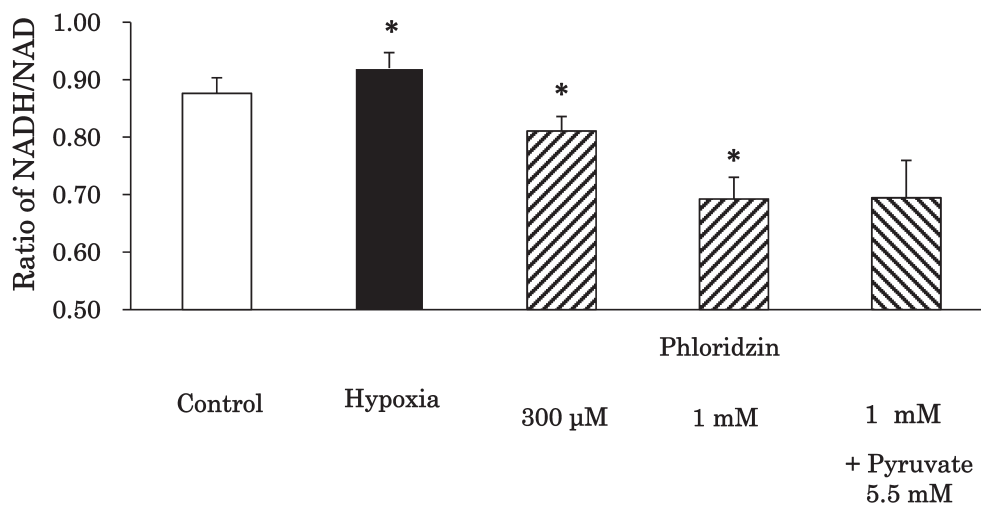


Fig. 3. Effect of phloridzin on ratio of NADH/NAD in rat ileum. Aeration N_2 or application of phloridzin was simultaneous with $H-65K^+$. All values were obtained 20 min after application of each reagent. The data are expressed as means \pm S.E.M. (N=4). * $P<0.05$; Significantly different from the $H-65K^+$ column (Control).

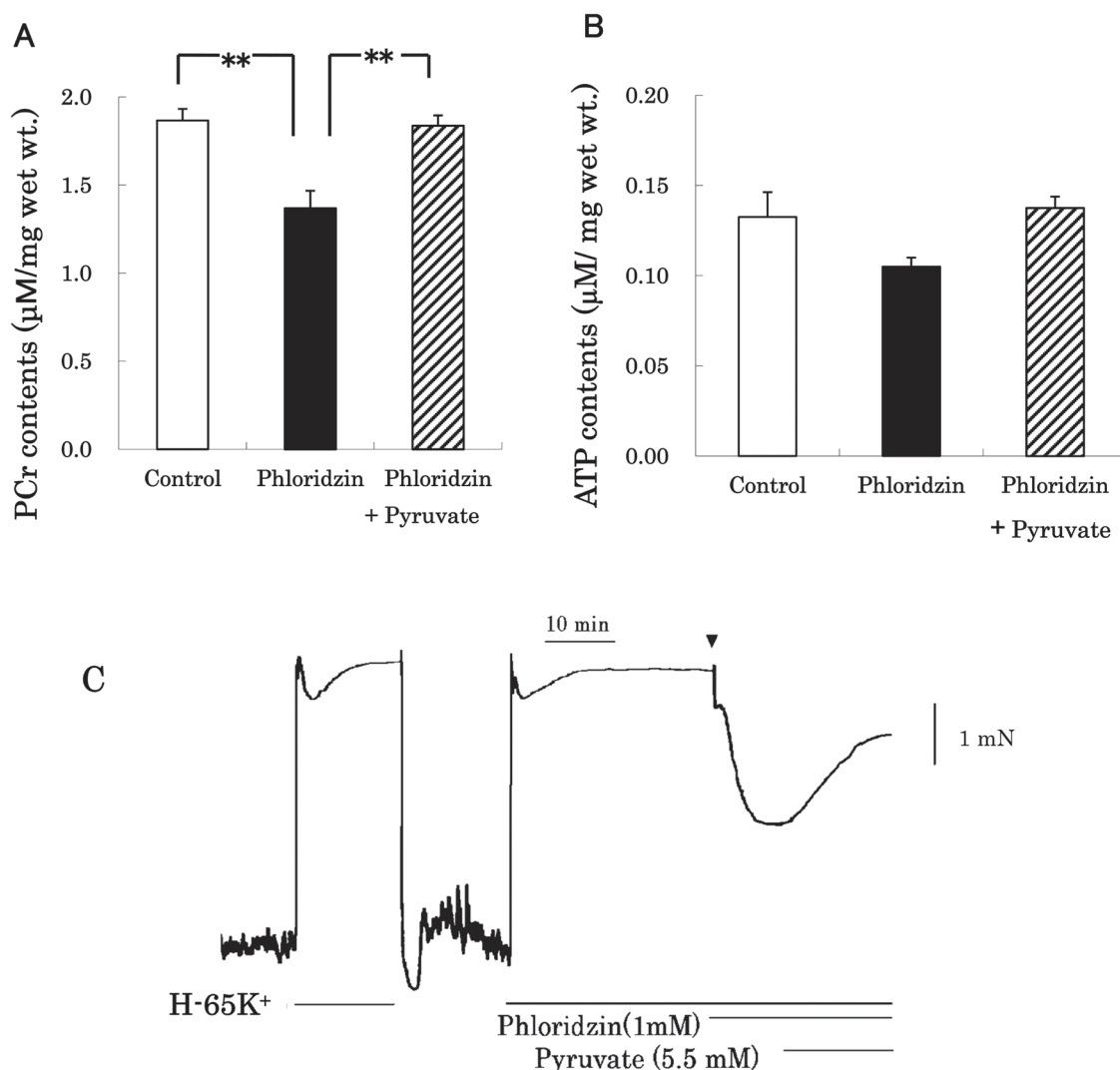


Fig. 4. Effects of phloridzin on phosphocreatine (PCr) (A) and ATP (B) contents. All PCr and ATP samples were treated with each reagent for 20 min. After the treatment, samples were frozen and measured. All conditions are treated with at least H-65K⁺. C is the typical trace of pyruvate-induced recovery of phloridzin-induced relaxation in presence of H-65K⁺. The data are expressed as means \pm S.E.M. (N=4). **Significantly different from the phloridzin column ($P < 0.01$).

Effects of phloridzin on high K⁺-induced increases in PCr and ATP contents in rat ileum

As shown in Fig. 4A, 1 mM phloridzin significantly reduced PCr contents of the ileum in the presence of H-65K⁺. Furthermore, 5.5 mM pyruvate recovered the reduction of PCr contents (Fig. 4A). In contrast, phloridzin and pyruvate did not affect ATP contents (Fig. 4B). Moreover, pyruvate recovered the phloridzin-induced inhibition of H-65K⁺-induced contraction in rat ileum (Fig. 4C).

Effect of phloridzin on glucose uptake in rat ileum smooth muscle cells

Isolated smooth muscle cells were immunostained by α -SMA and hoechst33342. Hoechst33342 and α -SMA double-positive cells were considered to be ileal smooth muscle cells (Fig. 5A). Application of H-65K⁺ increased 2-NBDG uptake significantly (Fig. 5B). However, isolated cells died from application of 300 μM and 1 mM phloridzin. Thus, concentrations of phloridzin were changed to 10 and 100 μM . The increase was inhibited by phloridzin (10 and 100 μM) in a concentration-dependent manner (Fig. 5B).

DISCUSSION

Based on the following observations, we have concluded that the inhibitory mechanism of phloridzin on high K⁺-induced contraction is due to the inhibition of glucose uptake via SGLT1 in rat ileum. (1) Phloridzin potently inhibited the high K⁺-induced contraction in the ileum to a greater degree than in the aorta. (2) The expression of SGLT1 mRNA on ileal smooth muscle was

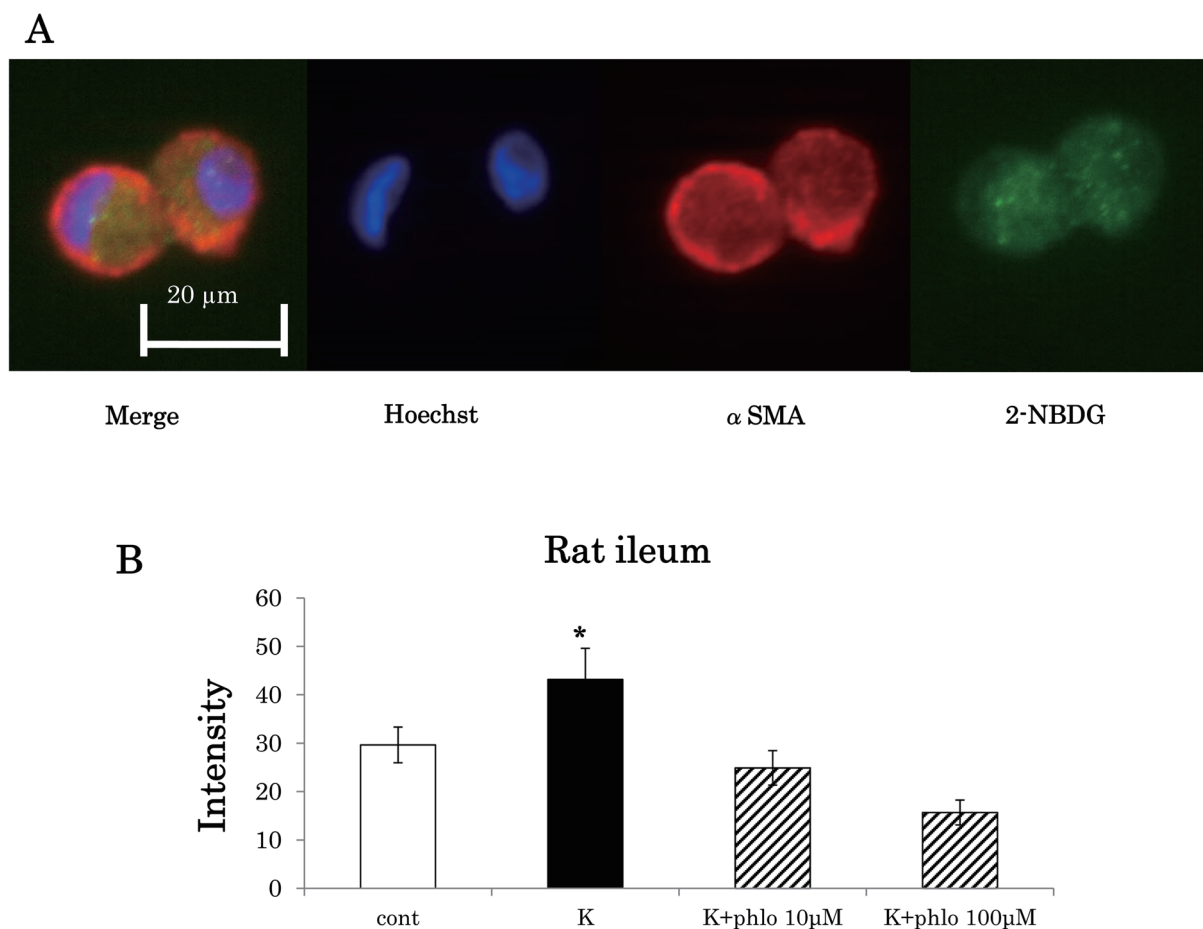


Fig. 5. Effect of phloridzin on fluorescent glucose uptake in rat ileum cells. A) The typical photo of immunostained cells. Blue fluorescence is nuclei, red is smooth muscle actin, and green is fluorescent glucose. B) Cells were treated with fluorescent glucose, 2-NBDG and each reagent for 20 min. Cells were observed 39 (cont), 36 (K), 48 (K+phlo 10 μ M) and 52 (K+phlo 100 μ M) cells in each condition. The data are expressed as means \pm S.E.M. (N=4). * P <0.05; ** P <0.01; Significantly different from the K column.

higher than that on the aorta. (3) In the presence of high K^+ , phloridzin reduced the ratio of NADH/NAD in the ileum in a concentration-dependent manner. (4) In the presence of high K^+ , phloridzin significantly reduced PCr contents. and (5) Phloridzin inhibited the high K^+ -induced increases of 2-NBDG uptake.

Enzymes of glycolysis pathway, tricarboxylic acid (TCA) cycle and electron transport chain exist in smooth muscle cells as well as other cells. Many researchers have utilized glucose for energy substrate in the contractile experiments of smooth muscle. However, to our knowledge, there are few reports that show the role of glucose transport on smooth muscle contraction. It was reported that ritonavir, a GLUT4 inhibitor, inhibited noradrenalin- and serotonin-induced contraction due to the inhibition of glucose uptake in mouse aorta, a tonic muscle [23]. On the other hand, application of an isosmotically substituted high K^+ , Na^+ -deficient solution induces contraction followed by gradual relaxation in various smooth muscles. The possible mechanisms of the relaxation are classified as three types: (1) swelling of the cells as in rabbit aorta [29], guinea pig trachea [26] and bovine trachea [12]; (2) inhibition of glucose uptake as in guinea pig urinary bladder [26] and guinea pig ileum [25]; and (3) the combination of swelling of cells and inhibition of glucose uptake as in guinea pig gallbladder [26], guinea pig tenia coli [6, 31] and porcine trachea [13]. Thus, these reports suggest that SGLTs play an important role in contraction of phasic muscle, such as urinary bladder and tenia coli. Moreover, phloridzin inhibited muscle contraction induced by high K^+ solution including glucose, but not that by the solution including pyruvate in guinea pig tenia coli [28]. In the present study, phloridzin inhibited high K^+ -induced muscle contraction in a concentration-dependent manner, and the inhibition was recovered by application of pyruvate in rat ileum. Furthermore, 1 mM phloridzin significantly inhibited high K^+ -induced muscle contraction in porcine iris sphincter. On the other hand, 1 mM phloridzin slightly inhibited high K^+ -induced muscle contraction in rat aorta and porcine renal artery. These results suggest that phloridzin inhibits high K^+ -induced muscle contraction due to the inhibition of SGLT, and the activation of SGLT may be important to keep muscle contraction in phasic muscle rather than tonic muscle. Moreover, the difference of the effect of phloridzin on high K^+ -induced contraction between phasic muscle and tonic muscle is probably due to the expression of SGLT1.

The expression of SGLT1 mRNA in rat ileum was significantly higher than that in the aorta. However, the expression of SGLT2

mRNA was the same level in the ileum and aorta. These results indicate that the phloridzin-induced inhibition of high K^+ -induced muscle contraction is related to SGLT1.

Hypoxic conditions increase the ratio of NADH/NAD, produced by glucose metabolism, in the cerebral cortex [7]. Moreover, hypoxic conditions increase the ratio of NADH/NAD in smooth muscle [20]. These data indicate that the ratio of NADH/NAD represents the activation of glycolytic pathway.

In the presence of high K^+ , phloridzin inhibited the ratio of NADH/NAD in a concentration-dependent manner in rat ileum. However, application of pyruvate did not affect the phloridzin-induced inhibition. These results suggested that the phloridzin inhibited high K^+ -induced muscle contraction due to the inhibition of SGLT1, resulting in the decrease of glucose uptake and glycolytic pathway.

In smooth muscle energy metabolism, ATP is generated from the glycolysis pathway, TCA cycle and electron transport chain, consequently phosphorylating creatine to produce PCr for energy storage. ATP required for smooth muscle contraction is supplied by the phosphorylation of ADP into ATP via creatine kinase dephosphorylation of stored PCr. In our study, phloridzin significantly decreased PCr contents, and the decrease was recovered by application of pyruvate in rat ileum. Moreover, application of pyruvate recovered phloridzin-induced muscle relaxation in rat ileum. However, phloridzin did not affect ATP contents, significantly. It has been reported that there is compartmentation of ATP synthesis and utilization in smooth muscle [11]. Further studies will clarify the relationship between muscle contraction and changes of ATP contents. These results strongly suggested that the phloridzin inhibited high K^+ -induced muscle contraction due to the inhibition of glycolytic pathway.

GLUT1 is widely distributed in normal tissues. However, the increases of uptakes in 2-NBDG, a fluorescent substrate, were sodium dependent, and the increases were inhibited by phloridzin in SGLT1- or SGLT2-expressed COS-7 cells [2] and HEK293 cells [15]. In our data, phloridzin inhibited high K^+ -induced contraction and 2-NBDG uptake in a concentration-dependent manner in rat ileum. These results suggested that the phloridzin inhibited high K^+ -induced muscle contraction due to the inhibition of glucose uptake via SGLT but not GLUT1.

In clinical trials of dual SGLT1 and SGLT2 inhibitors, such as sotagliflozin, there was not much evidence for gastrointestinal adverse effects in type 2 diabetes mellitus patients or in healthy subjects [34–36]. In the present study, phloridzin inhibited high K^+ -induced muscle contraction through inhibition of SGLT1. Thus, this study supplies the finding that inhibition of SGLT1-evoked inactivity of gastrointestinal motility may be related to adverse effects of dual SGLT1 and SGLT2 inhibitors, such as sotagliflozin.

In summary, phloridzin inhibited high K^+ -induced muscle contraction due to the inhibition of SGLT1, resulting in the decrease of glucose uptake in rat ileum. Moreover, this study indicates that phloridzin strongly inhibited high K^+ -induced muscle contraction in phasic muscle, such as the ileum, rather than tonic muscle, such as the aorta, and the difference is due to the expression of SGLT1.

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