Interaction of the Sodium/Glucose Cotransporter (SGLT) 2 Inhibitor Canagliflozin with SGLT1 and SGLT2: Inhibition Kinetics, Sidedness of Action, and Transporter-Associated Incorporation Accounting for its Pharmacodynamic and Pharmacokinetic Features

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ABSTRACT
Canagliflozin, a selective sodium/glucose cotransporter (SGLT) 2 inhibitor, suppresses the renal reabsorption of glucose and decreases blood glucose level in patients with type 2 diabetes. A characteristic of canagliflozin is its modest SGLT1 inhibitory action in the intestine at clinical dosage. To reveal its mechanism of action, we investigated the interaction of canagliflozin with SGLT1 and SGLT2. Inhibition kinetics and transporter-mediated uptake were examined in human SGLT1- or SGLT2-expressing cells. Whole-cell patch-clamp recording was conducted to examine the sidedness of drug action. Canagliflozin competitively inhibited SGLT1 and SGLT2, with high potency and selectivity for SGLT2. Inhibition constant (K_i) values for SGLT1 and SGLT2 were 770.5 and 4.0 nM, respectively. ^14C-canagliflozin was suggested to be transported by SGLT2; however, the transport rate was less than that of α-methyl-D-glucopyranoside. Canagliflozin inhibited α-methyl-D-glucopyranoside-induced SGLT1- and SGLT2-mediated inward currents preferentially from the extracellular side and not from the intracellular side. Based on the K_i value, canagliflozin is estimated to sufficiently inhibit SGLT2 from the urinary side in renal proximal tubules. The K_i value for SGLT1 suggests that canagliflozin suppresses SGLT1 in the small intestine from the luminal side, whereas it does not affect SGLT1 in the heart and skeletal muscle, considering the maximal concentration of plasma-unbound canagliflozin. Similarly, SGLT1 in the kidney would not be inhibited, thereby aiding in the prevention of hypoglycemia. After binding to SGLT2, canagliflozin may be reabsorbed by SGLT2, which leads to the low urinary excretion and prolonged drug action of canagliflozin.

Introduction

Under euglycemic conditions, glucose in the glomerular filtrate is completely reabsorbed in the proximal tubules of the kidney so that it is not excreted into urine. In the process of glucose reabsorption, glucose in the luminal fluid is taken up by tubular epithelial cells via sodium/glucose cotransporters (SGLTs) in the apical membrane and then leaves the epithelial cells through the facilitative glucose transporters in the basolateral membrane to the bloodstream (Wright et al., 2011). Under physiological conditions, the bulk of filtered glucose (~90%) is reabsorbed by SGLT2 (SLC5A2), a low-affinity high-capacity transporter in the proximal convoluted tubule (particularly in the S1 segment) (Kanai et al., 1994; You et al., 1995). The remaining filtered glucose (~10%) is reabsorbed by SGLT1 (SLC5A1), a high-affinity low-capacity transporter in the proximal straight tubule (particularly in the S3 segment) (Hediger et al., 1987; Lee et al., 1994). In the small intestine, SGLT1 is present in the apical membrane of epithelial cells and is responsible for the intestinal absorption of glucose and galactose (Wright et al., 2011). To target SGLT2 in renal proximal tubules, selective SGLT2 inhibitors have been developed as antidiabetic agents. These compounds inhibit the renal reabsorption of glucose to increase its urinary excretion, thereby lowering plasma glucose levels in an insulin-independent manner (Liang et al., 2012; Boyle and Wilding, 2013; Fujita and Inagaki, 2014; Peene and Benhalima, 2014).

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R.O. and L.W. contributed equally to this work.

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ABBREVIATIONS: AMG, α-methyl-D-glucopyranoside; 8-Br-cAMP, 8-bromo-cAMP; DMSO, dimethylsulfoxide; DOX, doxycycline; HBSS, Hank’s balanced salt solution; IAMG, α-methyl-D-glucopyranoside-induced current; IAMG/Cm, current density; Ki, inhibition constant; PCR, polymerase chain reaction; SGLT, sodium/glucose cotransporter.
Canagliflozin, a C-glucoside with a thiophene ring (Nomura et al., 2010), is the first selective SGLT2 inhibitor approved by the US Food and Drug Administration to improve glycemic control in individuals with type 2 diabetes mellitus (Elkinson and Scott, 2013). The European Medicines Agency also approved canagliflozin for this use in 2013. The administration of canagliflozin at a clinical dosage to patients with type 2 diabetes mellitus induces urinary glucose excretion at ~100 g/day, which would not increase the risk of hypoglycemia (Devineni et al., 2012, 2013; Iijima et al., 2015). In addition to anti-hyperglycemic action, canagliflozin, as a selective SGLT2 inhibitor, exhibits other favorable effects for the treatment of type 2 diabetes mellitus, including loss in body weight and hypotensive effects (Sha et al., 2011; Lavalle-Gonzalez et al., 2013). Among the selective SGLT2 inhibitors currently available for clinical use, a characteristic of canagliflozin is its modest SGLT1 inhibitory action in the intestine at clinical dosage (Grempler et al., 2012; Kuriyama et al., 2014). This is probably because of its relatively low SGLT2/SGLT1 selectivity as well as its relatively high clinical dosage, owing to its high plasma protein binding (Fujita and Inagaki, 2014; Devineni et al., 2015).

A major question regarding the mechanisms of action of SGLT2 inhibitors is the sidedness of action (i.e., whether these compounds inhibit SGLT2 from the extracellular side (luminal side) or from the cytoplasmic side). A canonical nonselective SGLT inhibitor, phlorizin, the parent compound of selective SGLT2 inhibitors, exhibits a ~600-fold lower inhibition constant ($K_i$) value on SGLT1 from the extracellular side than it does from the intracellular side (Eskandari et al., 2005). Phlorizin is freely filtered by the glomerulus and mainly excreted into urine, which is in line with the idea that the glomerular-filtrated phlorizin acts on SGLTs from the luminal side to increase urinary glucose excretion (Silverman, 2005). In contrast, canagliflozin exhibits high plasma protein binding (99%), and its urinary excretion is 1% or less of a single oral dosage (Inagaki et al., 2014; Devineni et al., 2015; Kinoshita and Kondo, 2015). These observations raised the possibility that canagliflozin enters epithelial cells of the renal proximal tubule through the basolateral membrane to exert its inhibitory effect on SGLT2 in the apical membrane from the cytoplasmic side. There is currently no experimental evidence for the sidedness of the action of clinically used selective SGLT2 inhibitors. In the study using TA-3404, a compound with a canagliflozin-related structure, it has been suggested that the SGLT2 inhibition occurs from the extracellular side (Ghezzi et al., 2014). In addition, we speculated that canagliflozin is not only an inhibitor of SGLT2, but also a substrate for SGLT2, and that SGLT2-mediated active transport, at least in part, explains the low urinary excretion rate observed with the use of canagliflozin.

In this study, to reveal the molecular mechanisms of action of canagliflozin, we investigated its interaction with SGLTs in vitro using cultured cell lines stably expressing human SGLT1 and SGLT2. We examined the concentration-dependent profiles of inhibition to determine the $K_i$ values of canagliflozin for each SGLT, the uptake of canagliflozin by SGLTs using radioactive $^{14}$C-canagliflozin, and the sidedness of the drug action using whole-cell patch-clamp recording.

**Materials and Methods**

**Chemicals.** Canagliflozin was synthesized by the Mitsubishi Tanabe Pharma Corporation, Medicinal Chemistry Laboratory (Saitama, Japan). $\alpha$-Methyl-$\beta$-glucopyranoside (AMG) was purchased from Sigma-Aldrich (St. Louis, MO). Phlorizin was obtained from TCI (Tokyo, Japan). $^{14}$C-Canagliflozin (18S)-1,5-anhydro-1-C-3-[5-(4-fluorophenylthiophen-2-yl)-2][4-(methyl-4-yl)methylpheno]-glucitol hemihydrate (2.15 GBq/mmol) was synthesized by Janssen Research & Development, LLC (Spring House, PA). $^{14}$C-AMG (methyl-$\alpha$-$\beta$-glucose-$^{14}$C(U)glucopyranoside) (10.69 GBq/mmol) was from PerkinElmer (Boston, MA). Blasticidin S was purchased from InvivoGen (San Diego, CA). S-Bromo-cAMP (8-BrcAMP) was obtained from Sigma-Aldrich. Penicillin-streptomycin solution, hygromycin B, G418 sulfate, and doxycycline (Dox) were from Wako Pure Chemical Industries (Osaka, Japan).

**Cell Lines.** For the construction of the tetracycline-inducible expression vector, a plasmid backbone of pJTI R4 DEST (except the region flanked by $\lambda$ integrase attR1 and attR2 sites (nucleotide position 3842–5545)), and a partial fragment of pcDNA4/TO (nucleotide position 232–1319: composed of cytomegalovirus promoter, tetracycline operator, multiple cloning sites, and bovine growth hormone polyadenylation site) were amplified by polymerase chain reaction (PCR) using Pwo Super Yield DNA Polymerase (Roche Diagnostics, Mannheim, Germany). The obtained two PCR products were then ligated together by homologous recombination using In-Fusion HD Cloning Kit (Clontech, Palo Alto, CA). The nucleotide sequences of the primer pairs were as follows: forward 5′-GGCTTGCGTACCCGGAAGGTGAGCTG-3′ and reverse 5′-GCGGTAATCATGTCATCTGTAGC-3′ for pJTI R4 DEST, and forward 5′-CACCGAGCCACCCAGATTGAGGCACTG-3′ and reverse 5′-GACCATGATACCCCGTCCCGAG-3′ for pcDNA4/TO, wherein the complementary sequences between the primer sets are indicated by capital letters.

The coding sequences of human SGLT1 (GenBank accession #AB463272) and SGLT2 (GenBank accession #NM_003041.3) were amplified by PCR from Flexi ORF SGLT1 Clone pF1KB8725 (Promega, Madison, WI) and from a full-length synthetic SGLT2 CDS clone (GenScript USA, Piscataway, NJ), respectively. The PCR products were integrated into the tetracycline-inducible expression vector (HindIII/XbaI linearized) by homologous recombination using In-Fusion HD Cloning Kit (Clontech). The nucleotide sequences of the primer pairs were as follows: forward 5′-ACTTAGAGGTGacaggagtcgagctgac-3′ and reverse 5′-TTAAACGCGCCCTCTTGAAGtgccagagttgctgct-3′ for SGLT1; and forward 5′-GTTAAAAACTT-TAGCTTcagggagtgagctgac-3′ and reverse 5′-AACAGCCCTCTAGTtagagctgagctgctgctgagctgac-3′ for SGL2, wherein the primer sequences identical to that of the tetracycline-inducible expression vector were indicated by capital letters.

To introduce an R4 int targeting site and promoter-less neomycin resistance gene into the genomic PhiC31 pseudo-attP site, the TetR-expressing T-Rex-CHO and T-Rex-293 cells (Invitrogen, Carlsbad, CA) were cotransfected with pJTI phiC31 Int and pJTI/Neo (Promega, Madison, WI) and from a full-length synthetic SGLT2 CDS clone (GenScript USA, Piscataway, NJ), respectively. The PCR products were integrated into the tetracycline-inducible expression vector (HindIII/XbaI linearized) by homologous recombination using Fusion HD Cloning Kit (Clontech). The nucleotide sequences of the primer pairs were as follows: forward 5′-ACTTAGAGGTGacaggagtcgagctgac-3′ and reverse 5′-TTAAACGCGCCCTCTTGAAGtgccagagttgctgct-3′ for SGLT1; and forward 5′-GTTAAAAACTT-TAGCTTcagggagtgagctgac-3′ and reverse 5′-AACAGCCCTCTAGTtagagctgagctgctgctgagctgac-3′ for SGL2, wherein the primer sequences identical to that of the tetracycline-inducible expression vector were indicated by capital letters.

To introduce an R4 int targeting site and promoter-less neomycin resistance gene into the genomic PhiC31 pseudo-attP site, the TetR-expressing T-Rex-CHO and T-Rex-293 cells (Invitrogen, Carlsbad, CA) were cotransfected with pJTI phiC31 Int and pJTI/Neo using X-tremEGENE 9 DNA Transfection Reagent (Roche Diagnostics). After the selection for hygromycin B resistance and cloning, the established Jump-In Ti platform cells (termed as JTRex-CHO and JTRex-293 cells) were further cotransfected with pJTI R4 Int and the tetracycline-inducible expression vectors for human SGLT1 or SGLT2 constructed as described above. Successful retargeting of the expression vectors at the R4 attP site confers neomycin resistance to the Jump-In Ti platform cells by cointegration of the EFla promoter upstream of the promoter-less neomycin resistance gene. Retargeted cells were selected for G418 sulfate resistance, cloned, and used for the measurements.

Cells were grown at 37°C in a humidified incubator supplied with 5% CO$_2$. Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 and DMEM (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (ThermoFisher Scientific, Grand Island, NY), 1% (v/v) penicillin-streptomycin solution, and 5 μg/ml blasticidin S were used to maintain the T-Rex-CHO and T-Rex-293 cells, respectively. Where indicated, hygromycin B (100 μg/ml) and G418 sulfate (500 μg/ml) were additionally supplied to the medium for selection and maintenance of the constructed stable cell lines.
14C-AMG Uptake Measurement and Inhibition Experiments. Uptake of AMG, an SGLT-selective substrate, was measured in the cell lines. Cells were seeded on 24-well plates and cultured for 24 hours in the presence of 10 ng/ml DOX to induce the expression of human SGLTs. Uptake measurements were performed as previously described (Wiriyasermkul et al., 2012). Briefly, cells were incubated at 37°C in glucose-free HBSS containing 14C-AMG at concentrations of 400 μM (25.4 MBq/mmol) and 1500 μM (50.8 MBq/mmol) for SGLT1 and SGLT2, respectively. NaCl in glucose-free HBSS was replaced with choline chloride for the sodium-free condition. For inhibition experiments, the indicated concentrations of phlorizin or canagliflozin dissolved in dimethylsulfoxide (DMSO) were added to the assay buffer. The assay buffer contained up to 0.1% DMSO (final concentration), unless otherwise noted. Up to 0.2% of DMSO did not significantly affect the uptake of 14C-AMG and 14C-canagliflozin (data not shown). After cell lysis, the radioactivity was measured by liquid scintillation counting and normalized according to the protein amount. The IC50 of phlorizin and canagliflozin was determined by fitting the data to inhibition curves using nonlinear regression (four-parameter Hill function; SigmaPlot 12.5; SYSTAT). To determine the kinetic properties of inhibition, uptake measurements were performed with varied concentrations of 14C-AMG in the presence or absence of the indicated concentrations of inhibitors. Uptake rates were plotted against 14C-AMG concentration and fitted to the Michaelis-Menten curve. Km was calculated using the following equation when inhibition was observed: V = Vmax/[1 + (Km/[S]) [1 + ([I]/Ki)], where V is the 14C-AMG uptake rate, Vmax is the maximal 14C-AMG uptake rate, Km is the Michaelis constant; [S] is the 14C-AMG concentration, and [I] is the inhibitor concentration. All analyses were performed using the enzyme kinetics module of SigmaPlot 12.5 (SYSTAT, San Jose, CA).

14C-Canagliflozin Uptake Measurements. For 14C-canagliflozin uptake measurement, cells were incubated with the indicated concentrations of 14C-canagliflozin (2.15 GBq/mmol) in the presence or absence of sodium. Phlorizin (50 μM) was added to the assay buffer where indicated. To examine the temperature dependence of the uptake, the measurements were performed using 14C-canagliflozin (5 and 10 nM) or 14C-AMG (500 μM, 25.4 MBq/mmol) for 20 minutes on ice or at 37°C.

Whole-Cell Patch-Clamp Recordings. Cells were seeded on poly-o-lysine–coated glass coverslips and cultured in the presence of DOX (10 ng/ml) for 12–20 hours. To increase the activity of SGLT2, 8-Br-cAMP (0.1 mM) was added to the medium 1 hour before the measurements (Ghezzi and Wright, 2012). Whole-cell patch-clamp recording was performed at room temperature for SGLT1 (JTREx293-SGLT1 cells) and at 32°C for SGLT2 (JTREx293-SGLT2 cells) in a stage-top chamber on an Eclipse TE500 inverted microscope (Nikon, Tokyo, Japan) with gravitational perfusion of external solution [100 mM Mannitol, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES-Tris (pH 7.4)]. To elicit the transporter-mediated current, the indicated concentration of AMG was added to the external solution, at which no significant inward currents were induced by the equivalent osmolality of mannitol (Supplemental Fig. 4). Patch pipettes were prepared from borosilicate glass capillary GD-1.5 (Narishige, Tokyo, Japan) pulled by a PD-7 pipette puller (Narishige), giving a resistance of 7–10 MΩ when filled with internal solution. The composition of the standard internal solution was 145 mM CsCl, 5 mM NaCl, 11 mM EGTA, and 10 mM HEPES-Tris (pH 7.4). A high-sodium internal solution [150 mM NaCl, 11 mM EGTA, and 10 mM HEPES-Tris (pH 7.4)] was used for the intracellular application of canagliflozin. Canagliflozin was dissolved in DMSO, and was diluted into internal and external solutions. External and internal solutions with and without canagliflozin contained 0.5% DMSO (final concentration), the concentration at which DMSO did not significantly affect the AMG/SGLT1 and SGLT2 mediations (Supplemental Fig. 4). The whole-cell currents were recorded at a holding potential of −60 mV using an AXOPATCH 200B amplifier (Axon Instruments, Foster City, CA) with a low-pass Bessel filter set at 5 kHz. Data were digitized at 10 kHz by a Digidata 1320A AD/DA converter (Axon Instruments) with Clampfit 10.4 software (Molecular Devices, Sunnyvale, CA) and analyzed using Clampfit 10.4 software (Molecular Devices). The whole-cell capacitance (Cm) was measured at the beginning of each measurement by the square-pulse method with the Membrane Test tool of the Clampex 8.2 software. The current density (AMG-induced current (IAMG)/Cm) was calculated to normalize the recorded IAMG.

To investigate the sidedness of inhibition by canagliflozin on SGLT1, the measurements were performed in the presence (n = 10) or absence (n = 11) of intracellular canagliflozin (20 μM). In the case of experiments on SGLT2, all the measurements were performed with intracellular application of canagliflozin (200 nM), and the IAMG/Cm values before and after the application of extracellular canagliflozin (200 nM) were compared (n = 7). The indicated concentrations of canagliflozin itself did not induce a significant current when applied extracellularly (Supplemental Fig. 4). Replacement of the intracellular fluid with high-sodium internal solution containing canagliflozin was confirmed under current-clamp mode (0 pA) after achieving whole-cell configuration.

Statistical Analyses. Unpaired, two-tailed Student’s t-tests were performed with Excel 2013 software (Microsoft, Redmond, WA), where p values < 0.05 were considered to be significant.

Results

Functional Expression of SGLT1 and SGLT2. To confirm the functional expression of SGLT1 and SGLT2, 14C-AMG uptake was measured in doxycycline-treated JTREx-CHO-SGLT1 and JTREx-CHO-SGLT2 cells. Doxycycline increased 14C-AMG uptake in the JTREx-CHO-SGLT1 and JTREx-CHO-SGLT2 cells but not in the parental JTREx-CHO cell line (Fig. 1, A and B). The expressed 14C-AMG uptake was Na+ dependent and was abolished in the absence of Na+. Phlorizin almost completely suppressed 14C-AMG uptake in both cell lines (Fig. 1, A and B). The 14C-AMG uptakes by SGLT1 and SGLT2 were linearly dependent on the incubation time for (at least) 30 and 90 minutes, respectively (Fig. 1, C and D). These results indicate that SGLT1 and SGLT2 were functionally expressed in the cell lines constructed. Protein expression of SGLT1 and SGLT2 was also confirmed by Western blot analysis using specific antibodies (Supplemental Fig. 1). Based on the linear range observed over the time course, the uptakes were measured for 5 and 30 minutes on SGT1 and SGLT2 cells, respectively, in the subsequent kinetic analyses.

Kinetic Properties of the Inhibition of 14C-AMG Uptake by Canagliflozin. The concentration dependence of the effects of canagliflozin on 14C-AMG uptake was examined and compared with that of the effects of phlorizin. Phlorizin inhibited 14C-AMG uptake mediated by SGLT1 and SGLT2 in a concentration-dependent manner (Fig. 2, A and B). The IC50 values of phlorizin on SGLT1 and SGLT2 were 439 and 56 nM, respectively. Similarly, the inhibition of SGLT1 and SGLT2 by canagliflozin also exhibited clear concentration dependence with an IC50 of 1550 nM for SGLT1 and 6.8 μM for SGLT2 (Fig. 2, C and D).

To further characterize the kinetic properties of the inhibitory effects of canagliflozin, the Ki values and mode of inhibition were determined. Nonlinear regression analysis of the inhibitory effects of the compounds (Supplemental Fig. 2, A, C, E, and G) revealed that canagliflozin and phlorizin competitively inhibit SGLT1- and SGLT2-mediated 14C-AMG uptake. The competitive inhibition was confirmed by Eadie-Hofstee plots (Supplemental Fig. 2, B, D, F, and H). The Ki values were determined from three or five independently performed trials, and are listed in Table 1.


**SGLT2-Mediated Uptake of 14C-Canagliflozin.** To examine whether canagliflozin is transported by SGLT1 and SGLT2, we measured the uptake of 14C-canagliflozin in the JTREx-CHO-SGLT1 and JTREx-CHO-SGLT2 cells. No statistically significant uptake of 14C-canagliflozin was detected for SGLT1 at 10 and 20 nM, although the JTREx-CHO-SGLT1 cells tended to exhibit a slightly higher uptake compared with mock cells in the presence of Na\(^+\) (Supplemental Fig. 3). In contrast, the JTREx-CHO-SGLT2 cells showed a significantly higher 14C-canagliflozin uptake compared with mock cells at 5–20 nM in the presence, but not in the absence, of Na\(^+\) (Fig. 3A). The considerable 14C-canagliflozin uptake observed in the parental JTREx-CHO cells most likely represents the incorporation via passive diffusion through the plasma membrane. SGLT2-mediated 14C-canagliflozin uptake increased in a concentration-dependent manner (Fig. 3A) and was completely inhibited by phlorizin (Fig. 3B), consistent with the properties of SGLT2-mediated transport. The uptake of 14C-canagliflozin via SGLT2 increased during the first 20 minutes of the incubation time (Fig. 3C).

To confirm that the SGLT2-associated increase of 14C-canagliflozin radioactivity is because of the SGLT2-mediated transport and not SGLT2 binding, its temperature dependency was examined. When similar measurements were performed on ice, uptake of the 14C-AMG in JTREx-CHO-SGLT2 cells decreased to the background level (i.e., to that of the parental JTREx-CHO cells), confirming the transporter-mediated uptake to be highly sensitive to temperature (Fig. 3D). Similarly, the JTREx-CHO-SGLT2 cells showed a higher 14C-canagliflozin uptake compared with the parental JTREx-CHO cells at 37°C, whereas this difference disappeared on ice (Fig. 3D). Such temperature dependence suggests that the observed 14C-canagliflozin uptake represents SGLT2-mediated transport but not the binding of 14C-canagliflozin to SGLT2.

**Fig. 1.** Properties of 14C-AMG uptake in the JTREx-CHO-SGLT1 and JTREx-CHO-SGLT2 cells. (A and B) Na\(^+\) dependence and phlorizin sensitivity of doxycycline-induced 14C-AMG uptake. Doxycycline-treated (Dox+) or untreated (Dox-) cells were used to determine 14C-AMG uptake in the presence or absence of Na\(^+\) and phlorizin (100 \(\mu\)M). For the JTREx-CHO-SGLT1 cells (SGLT1) and parental JTREx-CHO cells (Mock), the uptake of 14C-AMG (400 \(\mu\)M) was measured for 5 minutes (A). For the JTREx-CHO-SGLT2 cells (SGLT2) and parental JTREx-CHO cells (Mock), the uptake of 14C-AMG (1500 \(\mu\)M) was measured for 30 minutes (B). (C and D) Time course of 14C-AMG uptake examined in the presence of Na\(^+\) (glucose-free HBSS). 14C-AMG of 400 \(\mu\)M was used for the Dox-treated JTREx-CHO-SGLT1 (SGLT1) and parental JTREx-CHO (Mock) cells (C). 14C-AMG of 1500 \(\mu\)M was used for the Dox-treated JTREx-CHO-SGLT2 cells (SGLT2) and parental JTREx-CHO cells (Mock) (D). Data shown depict the mean ± S.E.M. n = 3–4. ***p < 0.001.
Sidedness of Canagliflozin Action. The sidedness of canagliflozin action on SGLTs was investigated using the whole-cell configuration of the patch-clamp method by applying canagliflozin to the outside or inside of the cells (Fig. 4A). Because AMG-induced electric currents in the JTREx-CHO-SGLT2 cells used for the uptake experiments were too small for reliable electrophysiological recording, we prepared other stable cell lines (JTREx-293-SGLT1 and JTREx-293-SGLT2 cells) established from the JTREx-293 cells used for whole-cell patch-clamp recording. When the AMG uptakes were measured in the two SGLT2-expressing cell lines, JTREx-293-SGLT2 cells showed a 2.32-fold higher uptake at 30 minutes than JTREx-CHO-SGLT2 cells (data not shown). Furthermore, to enhance the activity of SGLT2, 8-Br-cAMP was added to the medium, and measurements were conducted at 32°C (Hummel et al., 2011; Ghezzi and Wright, 2012).

For the intracellular application of the drug, the time course for the replacement of intracellular fluid with a canagliflozin-containing pipette solution was estimated under current-clamp mode (0 pA). After attaining the whole-cell configuration, the original inside-negative membrane potential was promptly shifted toward 0 mV, as expected from the ionic composition of the internal solution and reached a plateau at ~150 seconds (Supplemental Fig. 5), indicating complete replacement of intracellular fluid with the drug-containing pipette solution within this time range. Therefore, all the measurements were performed after 150 seconds.

Neither the JTREx-293-SGLT1 nor the JTREx-293-SGLT2 cells, without induction by doxycycline treatment, showed any significant background inward current by the addition of AMG into the external solution (Fig. 4B). After the

**TABLE 1**

<table>
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<tr>
<th>Inhibitor</th>
<th>$K_i$ for SGLT1 (nM)</th>
<th>$K_i$ for SGLT2 (nM)</th>
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<tr>
<td>Phlorizin</td>
<td>545.7 ± 56.89 (n = 3)</td>
<td>102.3 ± 27.41 (n = 3)</td>
</tr>
<tr>
<td>Canagliflozin</td>
<td>770.5 ± 85.19 (n = 5)</td>
<td>4.0 ± 0.77 (n = 3)</td>
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Values are presented as the mean ± S.E.M.
doxycycline treatment, a concentration of 10 mM AMG in the external solution induced an inward current in the JTREx-293-SGLT1 cells (Fig. 4C). This AMG-induced inward current was reverted to the basal level when either AMG or Na⁺ was washed out from the external solution during the measurement (Supplemental Fig. 4). The AMG-induced current was almost completely suppressed by extracellular application of 20 μM canagliflozin (Fig. 4C). In contrast, when the same concentration of canagliflozin was applied intracellularly, no significant inhibition of the SGLT1-mediated AMG-induced current was observed (Fig. 4E). Similarly, an external solution of 20 mM AMG induced an appreciable inward current in the JTREx-293-SGLT2 cells in the presence of intracellular 200 nM canagliflozin. This inward current was almost completely reverted to the basal level when the same concentration of canagliflozin was extracellularly applied (Fig. 4D). Figure 4F shows the comparison of the current densities of the AMG-induced current before and after the addition of extracellular canagliflozin, indicating that extracellular canagliflozin remarkably reduces AMG-induced currents. Taken together, these results demonstrate that canagliflozin preferentially inhibits SGLT1 and SGLT2 from the extracellular side.

Discussion

We performed a kinetic analysis of the inhibitory effect of canagliflozin and confirmed its high potency and selectivity for SGLT2 (Fig. 2 and Supplemental Fig. 2). This is the first report to describe the Ki values of canagliflozin, which are useful to evaluate the in vivo action of canagliflozin on SGLT2 and SGLT1 in various organs. Canagliflozin competitively inhibited human SGLT1 and SGLT2 with ~200-fold higher selectivity for SGLT2 than for SGLT1, based on the ratio of Ki values (Table 1). Based on the Ki values, canagliflozin has an ~25-fold higher affinity for SGLT2 than for phlorizin, whereas both inhibitors exhibited similar affinity for SGLT1 (Table 1). These results are in line with those of previous reports, wherein the potency and selectivity of canagliflozin were discussed based on IC50 values (Nomura et al., 2010; Gremler et al., 2012).

The whole-cell patch-clamp method allowed us to control the composition of intracellular as well as extracellular fluid, enabling the delivery of canagliflozin into the cells for examining the sidedness of the inhibition (Fig. 4). We demonstrated that canagliflozin preferentially inhibits SGLT2 from the extracellular side and not from the intracellular side. This suggests that canagliflozin acts on SGLT2

![Fig. 3. 14C-canagliflozin uptake mediated by SGLT2. (A and B) The measurement of 14C-canagliflozin uptake by SGLT2. The uptake of 14C-canagliflozin (5, 10, and 20 nM) was measured for 20 minutes in the JTREx-CHO-SGLT2 (SGLT2) and parental JTREx-CHO (Mock) cells with or without Na⁺ (A) and phlorizin (50 μM) (B). (C) Time course of 14C-canagliflozin uptake by SGLT2. The uptake of 5 nM 14C-canagliflozin was measured in the JTREx-CHO-SGLT2 (SGLT2) and parental JTREx-CHO (Mock) cells in the presence of Na⁺. (D) Temperature dependence of 14C-canagliflozin uptake by SGLT2. The uptakes of 14C-canagliflozin (5 and 10 nM) (left) and 14C-AMG (500 μM) (right) were measured either on ice or at 37°C for 20 minutes in the presence of Na⁺ using the Dox-treated JTREx-CHO-SGLT2 (SGLT2) and parental JTREx-CHO (Mock) cells. Data shown depict the mean ± S.E.M. n = 3–6. *p < 0.05; **p < 0.01; ***p < 0.001.](image-url)
Fig. 4. Sidedness of canagliflozin action on SGLTs. (A) Schematic representation of whole-cell recording of transporter-mediated AMG-induced electric currents. JTReX-293-SGLT1 and JTReX-293-SGLT2 cells were subjected to whole-cell patch-clamp recording at a holding potential of −60 mV. (B) The current trace of whole-cell recording demonstrating the absence of the background AMG-induced current in JTReX-293-SGLT1 (top) and JTReX-293-SGLT2 (bottom) cells without the induction by doxycycline. AMG (10 mM for SGLT1 and 20 mM for SGLT2) was added to the external solution. The traces of AMG-induced currents showing the inhibitory effect after extracellular application of canagliflozin on SGLT1 (C) and SGLT2 (D). AMG (10 mM for SGLT1 and 20 mM for SGLT2) and canagliflozin (20 μM for SGLT1 and 200 nM for SGLT2) were added to the external solution as indicated.
from the luminal side because SGLT2 is localized on the apical membrane of proximal tubules (Wright et al., 2011). Because the plasma protein binding of canagliflozin is ~99% (Devineni et al., 2015) and the maximal plasma concentration after oral administration of clinical doses is 2.1–6.14 μM (Kinoshita and Kondo, 2015; Sha et al., 2015), the maximal concentration of unbound canagliflozin in plasma and in the glomerular filtrate is ~20–60 nM. SGLT2 should be exposed to this approximate canagliflozin concentration from the luminal side. This is above the determined \( K_i \) value of canagliflozin for SGLT2 (4.0 nM), suggesting that canagliflozin fully inhibits SGLT2 from the luminal side. In the concentration range of 20–60 nM, canagliflozin is estimated to inhibit SGLT2 by 68–86% under euglycemic conditions [100 mg/dl (5.6 mM) \( \text{d-glucose} \)] and by 49–74% when the blood glucose level is elevated to 300 mg/dl (16.7 mM); this is based on the following equation: 

\[
V = V_{\max} / [1 + (K_m/[S]) (1 + (I/K_i))],
\]

where \( V \) is the uptake rate for \( \text{d-glucose} \), \( V_{\max} \) is the maximal uptake rate, \( K_m = 4.0 \text{mM} \) for \( \text{d-glucose} \) (Lu et al., 2014), \( [S] \) is the \( \text{d-glucose} \) concentration, \( I \) is the unbound canagliflozin concentration, and \( K_i = 4.0 \text{nM} \). This supports the idea that canagliflozin inhibits the renal tubular SGLT2 by acting from the luminal side but not from the basolateral side.

Previous studies demonstrated that the oral administration of canagliflozin delays postprandial glucose absorption from the intestine and enhances glucose-induced glucagon-like peptide-1 secretion (Polidori et al., 2013; Kinoshita and Kondo, 2015; Sha et al., 2015). Because other SGLT2 inhibitors with higher selectivity to SGLT2 have not been reported to elicit such effects, transient inhibition of SGLT1 in the upper small intestine seems to be the reason underlying the effect on intestinal glucose absorption (Polidori et al., 2013; Oguma et al., 2015a,b). Like SGLT2, we showed that SGLT1 is preferentially inhibited by canagliflozin from the extracellular side, with a \( K_i \) value of 770.5 nM. Because of the high plasma protein binding ratio (Devineni et al., 2015), the clinical dosage of canagliflozin is higher (100–300 mg) than other SGLT2 inhibitors. Therefore, it is reasonable to suppose that the luminal concentration of canagliflozin in the upper small intestine is transiently elevated after oral administration to a level considerably higher than the \( K_i \) value for SGLT1, implying that canagliflozin could inhibit SGLT1 from the luminal side of the intestine. The intraluminal concentration of canagliflozin in the small intestine is substantially elevated after oral administration (Oguma et al., 2015b).

In a survey of the expression of SGLTs in human tissues by quantitative PCR, SGLT1 was expressed at high levels in the heart and skeletal muscle, in addition to the small intestine (Chen et al., 2010). In the heart, SGLT1 mRNA was detected in cardiomyocytes by in situ hybridization (Zhou et al., 2003), and SGLT1 protein was localized to the cardiac myocyte sarcolemma (Banerjee et al., 2009). SGLT1 expression levels in cardiomyocytes by in situ hybridization (Zhou et al., 2003), and SGLT1 mRNA was detected in the heart, SGLT1 mRNA was detected in cardiomyocytes by in situ hybridization (Zhou et al., 2003), and SGLT1 protein was localized to the cardiac myocyte sarcolemma (Banerjee et al., 2009). SGLT1 expression levels in cardiomyocytes by in situ hybridization (Zhou et al., 2003), and SGLT1 mRNA was detected in the heart, SGLT1 mRNA was detected in cardiomyocytes by in situ hybridization (Zhou et al., 2003), and SGLT1 protein was localized to the cardiac myocyte sarcolemma (Banerjee et al., 2009). Although the functional roles of SGLT1 have not been determined in such nonepithelial tissues, it has been reported that SGLT1 is involved in the pathophysiology of a murine model of PRKAG2 cardiomyopathy (Ramratnam et al., 2014). Because the maximal plasma level of unbound canagliflozin in clinical studies (20–60 nM, see above) is ~1/30 to 1/10 of the \( K_i \) value for SGLT1 (770.5 nM), the oral administration of clinical doses of canagliflozin would not inhibit SGLT1 in the heart or skeletal muscle. In the concentration range of 20–60 nM, it is estimated that canagliflozin inhibits SGLT1 by 0.2–0.7% under euglycemic conditions [100 mg/dl (5.6 mM) \( \text{d-glucose} \) and by 0.1–0.2% when the blood glucose level is elevated to 300 mg/dl (16.7 mM); this is based on the assumption that the \( K_m \) for \( \text{d-glucose} \) is 0.5 M in SGLT1 (Lu et al., 2014). Similarly, oral administration of canagliflozin at clinical dosage would not inhibit SGLT1 in the proximal straight tubules of the kidney, aiding in the prevention of hypoglycemia in conjunction with the paradoxical increase in endogenous glucose production caused by SGLT2 inhibition (Abdul-Ghani et al., 2013; Cefalu, 2014).

Pharmacokinetic studies have revealed that the excretion of canagliflozin in urine is <1% of the administered dose (Inagaki et al., 2014; Devineni et al., 2015; Kinoshita and Kondo, 2015). We showed that canagliflozin is incorporated into the SGLT2-expressing cells by SGLT2-mediated transport in addition to passive diffusion through the plasma membrane (Fig. 3). Because the exogenous expression of SGLT2 in conventional expression systems is, for an unknown reason, generally low in efficiency (Canai et al., 1994), the uptake of \( ^{14}\text{C}-\text{canagliflozin} \) by SGLT2 was low (Fig. 3). Therefore, based on the data obtained, it is difficult to estimate the contribution of the possible SGLT2-mediated reabsorption of canagliflozin to total tubular canagliflozin reabsorption, with the latter also involving the passive diffusion of the compound through the renal tubule because of its high hydrophobicity. We propose that the tubular reabsorption of canagliflozin involving SGLT2-mediated transport and the low filtration rate because of its high plasma protein binding contribute to the low urinary excretion of canagliflozin. Moreover, a local circulation of canagliflozin through the apical membrane of renal proximal tubules may be facilitated by the SGLT2-mediated reabsorption of canagliflozin with the possible involvement of its efflux transporters. This would retain canagliflozin in the proximity of SGLT2, enabling the recurrent inhibition of SGLT2 from the luminal side, which explains the prolonged drug action beyond the decrease in blood canagliflozin levels (Inagaki et al., 2014; Kuriyama et al., 2014). The significance of SGLT2-mediated canagliflozin uptake in pharmacodynamics as well as pharmacokinetics in vivo remains to be investigated.

\( ^{14}\text{C}-\text{canagliflozin} \) uptake was not detected for SGLT1 over the concentration range tested (Supplemental Fig. 3). Higher concentrations of \( ^{14}\text{C}-\text{canagliflozin} \) could not to be examined because of a high background cellular incorporation. However, considering its lower affinity to SGLT1, it is important to test the uptake of canagliflozin by SGLT1 at a higher concentration using high-expression systems.
In conclusion, canagliflozin inhibited SGLT2 and SGLT1 in a competitive manner with high potency and selectivity for SGLT2. Canagliflozin acted from the extracellular side, suggesting that the inhibition occurs from the urinary side in renal proximal tubules. We also suggested that canagliflozin is, at least in part, transported by SGLT2 after binding, which may contribute to its pharmacodynamic and pharmacokinetic characteristics.

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