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Membrane topology of loop 13–14 of the Na^+/glucose cotransporter (SGLT1): A SCAM and fluorescent labelling study

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Abstract

The accessibility of the hydrophilic loop between putative transmembrane segments XIII and XIV of the Na^+/glucose cotransporter (SGLT1) was studied in *Xenopus* oocytes, using the substituted cysteine accessibility method (SCAM) and fluorescent labelling. Fifteen cysteine mutants between positions 565 and 664 yielded cotransport currents of similar amplitude than the wild-type SGLT1 (wtSGLT1). Extracellular, membrane-impermeant $MTSES^{(-)}$ and $MTSET^{(+)}$ had no effect on either cotransport or Na^+ leak currents of wtSGLT1 but 9 mutants were affected by MTSES and/or MTSET. We also performed fluorescent labelling on SGLT1 mutants, using tetramethylrhodamine-5-maleimide and showed that positions 586, 588 and 624 were accessible. As amino acids 604 to 610 in SGLT1 have been proposed to form part of a phlorizin (Pz) binding site, we measured the K_i^{Pz} and $K_m^{\alpha MG}$ for wtSGLT1 and for cysteine mutants at positions 588, 605–608 and 625. Although mutants A605C, Y606C and D607C had slightly higher K_i^{Pz} values than wtSGLT1 with minimal changes in $K_m^{\alpha MG}$, the effects were modest and do not support the original hypothesis. We conclude that the large, hydrophilic loop near the carboxyl terminus of SGLT1 is thus accessible to the external solution but does not appear to play a major part in the binding of phlorizin.

Keywords: Na^+/glucose cotransporter; Electrophysiology; SCAM; Fluorescence; Topology

1. Introduction

Despite their important physiological roles, little is known about the three-dimensional structures of membrane proteins and less than 35 different protein folds have been reported for transporters, channels or pumps (http://www.blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). In the absence of physical structure, an important step in our understanding of their mechanisms of action at the molecular level is to correctly identify the orientation of each protein segment with respect to the membrane. In the case of the Na^+/glucose cotransporter SGLT1[1], an archetype for Na^+ cotransporters, a proposed membrane topology posits extracellular N- and C-termini and 14 transmembrane segments (TMS) [1] comprising 48% of the protein. This model is based on hydrophobicity plots and on the insertion of glycosylation sites at different locations along the protein.

Several studies employing site-directed mutagenesis and chimeric constructions of SGLT1 have sought to identify the amino acids forming the Na^+ binding sites, the glucose binding site and the voltage sensor responsible for the charge displacement observed in the absence of glucose. Since expression of a truncated protein composed of TMS 10–13 of

Abbreviations: SGLT1, high affinity Na^+/glucose cotransporter; hSGLT1, human isoform of SGLT1; rSGLT1, rabbit isoform of SGLT1; wtSGLT1, wild-type SGLT1; SCAM, substituted cysteine accessibility method; MTS, methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)-methanethiosulfonate; MTSET, [2-(Trimethylammonium) ethyl]-methanethiosulfonate bromide; MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide; TMR5(6)M, tetramethylrhodamine-5(or 6)-maleimide; TMS, transmembrane segment; Pz, phlorizin; αMG, α−Methyl-glucose; V_m, membrane potential; I_{cotr}, αMG cotransport current; I_{leak}, Na^+ leak current; AA, amino acid; $K_m^{\alpha MG}$, apparent affinity for αMG; K_i^{Pz}, inhibition constant of Pz; K_m^{Na+}, apparent affinity for Na^+; VSVG, vesicular stomatitis virus G protein

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SGLT1 in *Xenopus* oocytes is associated with an increase in glucose permeability, it has been suggested that the C-terminal end of SGLT1 forms the glucose permeation pathway [2,3]. However, this permeation pathway does not appear to be specific for glucose as mannitol fluxes were also increased and Pz sensitivity was lost [3]. Replacing residue Q457 with a cysteine in this C-terminal portion of SGLT1 and labelling it with a fluorophore (tetramethylrhodamine-6-maleimide, TMR6M) showed that this residue is involved in a voltage-dependent conformational change that affects the fluorescence of TMR6M by 4–5% [4,5]. The putative loop between TMS 4 and 5 has been proposed to form part of the Na^+ interaction and voltage sensing domain based on an interpretation of the effects on pre-steady state currents of mutation of a series of amino acids there to cysteine residues +/− thiol-binding reagents [6–9]. The evidence supporting the physical separation between the putative pathways used by Na^+ and glucose appears questionable especially when compared to the recent crystallographic structure of the H^+/lactose cotransporter [10].

An extremely hydrophilic segment of 88 amino acids positioned between the 13th and 14th TMS (amino acids (AA) #549 to 637 in the human clone) is considered to be intracellular by the model described above. This region is also noteworthy because it contains a quite conserved sequence followed by a less conserved one when the SGLT1 protein sequence is aligned with the closely related SGLT2 and SGLT3 proteins (see Fig. 1A). The location of this segment, which contains 37 charged amino acids (21 negatively charged and 16 positively charged), was later challenged by Kinne's group based on the observation that histidine tags inserted at positions 584–588 and 622–627 can be recognized by an antibody in the external solution [11]. More recently, the segment between 604 and 610 has also been proposed to be involved in the extracellular binding site for phlorizin (Pz), a specific inhibitor for SGLT1 [12–14].

As a step in establishing the structure and function of loop 13–14, we report here our observations of the electrophysiological characterization and fluorescent labelling of 15 different cysteine mutants created between residues N565 and A664 within the last hydrophilic loop and the last TMS of human SGLT1 expressed in *Xenopus* oocytes. The results clearly indicate that at least 10 of these 15 mutants are accessible from the extracellular solution by membrane-impermeant reagents or with a rhodamine-based fluorophore (tetramethylrhodamine-5-maleimide, TMR5M). In addition, mutations at positions 605–608 suggest a weak role for this segment in the ability of SGLT1 to bind extracellular Pz.

2. Materials and methods

2.1. Molecular biology

We used human SGLT1 cDNA with an N-terminal myc epitope in the expression vector pBS (pBS-myc-hSGLT1) [15] to construct each of the mutations described here. They were created using the method described by Fisher and Pei [16]. Individual mutations were confirmed by DNA sequencing and the specific amino acids which were mutated to cysteine are indicated in Fig. 1B. QIAGEN mini-prep kits were used to extract DNA and *Eco*RI digestion was followed by mRNA transcription in vitro using the T3 mMessage mMachine kit (Ambion, Austin TX).

2.2. Oocyte preparation and injection

Oocytes were surgically removed from *Xenopus laevis* frogs, dissected and defolliculated as described previously [15,17]. They were injected with 46 nl mRNA (0.25 μg/μl), aside from the wild-type SGLT1 (wtSGLT1), which was injected with 46 nl of a 0.1 μg/μl solution, and maintained in Barth's solution (in mM: 90 NaCl, 3 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$, 5 HEPES, pH 7.6) supplemented with 5% horse serum, 2.5 mM Na^+ pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin for 4–8 days prior to experimental use.

2.3. Electrophysiology

The saline solution is composed of (in mM): 90 NaCl, 3 KCl, 0.82 $MgCl_2$, 0.74 $CaCl_2$, 10 HEPES, and the solution pH was adjusted to 7.6 with NaOH. Steady-state currents were measured with 5 mM α-Methyl-glucose (αMG) and 200 μM phlorizin (Pz) for all experiments except for determination of apparent affinity for αMG ($K_m^{\alpha MG}$) and inhibition constant of Pz (K_i^{Pz}), which were done as described previously [15]. Two-microelectrode voltage clamp experiments were performed with an Oocyte Clamp OC-725 (Warner Instrument Corp, Hamden, CT). Three repetitions of membrane potential steps (25 mV or 20 mV) between + 70 and −170 mV were imposed for 300 ms intervals per step. Data were obtained with a sampling frequency of 10 kHz (V was filtered at 1 kHz lowpass), and the 3 repetitions were averaged during the experiment by the acquisition program (Clampex 8.2.0.228, Axon Instruments Inc, Union City, CA). Steady-state current levels were obtained using mean values for measurements at the end of each pulse (between 150 and 300 ms). Treatments with 2.5 mM methanethiosulfonate (MTS) reagents were performed at the normal holding potential (−50 mV) in a saline solution for 5 to 10 min; afterwards, the bath was washed sufficiently to eliminate unbound reactants. The activity of the cotransporter was tested before and after the treatment. The effects of treatments on the different mutants were normalized by dividing currents measured after treatment with the currents measured before treatment; these were compared to wtSGLT1 currents, normalized in the same fashion.

2.4. Fluorescence

A Nikon Diaphot inverted microscope was employed with a 40× oil immersion objective (numerical aperture