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Note

Competitive Inhibition of AQP7-mediated Glycerol Transport by Glycerol Derivatives

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Summary: Aquaporin 7 (AQP7) is an aquaglyceroporin that has recently been found to operate as a facilitative carrier rather than a channel for glycerol, although its primary function is as a water channel. To probe into its substrate specificity, we examined the inhibitory effect of a series of acyl glycerol derivatives on glycerol transport mediated by human AQP7 stably expressed in Madin-Darby canine kidney II cells. According to kinetic analyses, AQP7-mediated glycerol transport was found to be competitively inhibited by monoacetin, monobutyrin and diacetin. Therefore, it may be possible that they all could be recognized as substrates by AQP7. The inhibition constant (K_i) of monoacetin (134 μM) was smaller than that of diacetin (420 μM), but greater than the Michaelis constant for glycerol (11.8 μM). Considering another finding that inhibition by triacetin was insignificant, it is likely that a decrease in the number of hydroxyl groups in the glycerol molecule by acetyl derivatization leads to a decrease in affinity for AQP7. The K_i of monobutyrin (80 μM) was, on the other hand, comparable with that of monoacetin, suggesting that the extension of the acyl chain by two hydrocarbon units does not have an impact on affinity for AQP7.

Keywords: aquaporin 7; facilitative carrier; glycerol; glycerol derivatives; competitive inhibition; substrate specificity

Introduction

Aquaporins (AQPs), which have been primarily characterized as water channels, include a group of members that can also transport glycerol and are called aquaglyceroporins.[1,2] Although aquaglyceroporins have generally been assumed to operate in a channel mode, which is of nonsaturable nature, for glycerol as they do for water, a series of our studies on AQP9,[3] AQP10[4] and, most recently, AQP7[5] revealed that these aquaglyceroporins operate in a carrier mode, which is of saturable nature, for glycerol and brought them to attention for the possibility that they might be involved in the transport of glycerol derivatives and analogous drugs. However, the substrate specificity of these aquaglyceroporins is mostly unknown yet.

We here report our attempt to help clarify the substrate specificity of AQP7, which is mainly expressed in the fat.[6,7] It was suggested in our recent study that, based on the assessment of the inhibition of AQP7-mediated glycerol transport by several compounds analogous to glycerol, the 1,2-diol structure with 2- or 3-carbon backbone may play an important role in recognition by AQP7.[5] This characteristic is shared by AQP9 and AQP10, but no information has so far been available beyond that. Therefore, as an extension of our study based on the suggestion, we mainly examined the effect of mono-, di- and tri-acyl derivatives of glycerol on AQP7-mediated glycerol transport in the present study to focus on the role of hydroxyl groups in recognition by AQP7.

Materials and Methods

Chemicals: [2-^{3}H(N)]Glycerol (29.6 GBq/mmol) was obtained from PerkinElmer (Boston, MA). Unlabeled glycerol and Dulbecco's modified Eagle's medium (DMEM) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). All other reagents were of analytical grade and commercially available.

Cell culture: Madin-Darby canine kidney II (MDCKII) cells were maintained at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

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Preparation of MDCKII cells stably expressing AQP7: The cDNA of human AQP7 was cloned from the human testis total RNA (Clontech, Mountain View, CA) by reverse transcription and subsequent polymerase chain reaction, incorporated into pCI-neo vector (Promega, Madison, WI) to prepare a plasmid for transfection and stably transfected into MDCKII cells, as described previously.[5)]

Uptake study: MDCKII cells stably expressing AQP7 (2×10^5 cells/1 mL/well initially) were grown on 24-well plates for 48 h to confluence. The cells in each well were preincubated in 1 mL of substrate-free uptake buffer, that is, Hanks' solution (136.7 mM NaCl, 5.36 mM KCl, 0.952 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.441 mM KH_2PO_4, 0.385 mM Na_2HPO_4 and 25 mM D-glucose) supplemented with 10 mM HEPES (pH 7.4), for 5 min and uptake assays were started by replacing the substrate-free uptake buffer for preincubation with uptake buffer containing [^{3}H]glycerol (0.25 mL). Glycerol derivatives were included in the uptake buffer in assays to test their respective inhibitory effects. Assays were stopped by addition of ice-cold substrate-free uptake buffer (2 mL) and the cells were washed two times with 2 mL of the same buffer. The cells were solubilized in 0.5 mL of 0.2 M NaOH solution containing 0.5% sodium dodecyl sulphate at room temperature for 1 h and the associated radioactivity was measured by liquid scintillation counting, using 3 mL of Clear-sol I (Nacalai Tesque, Kyoto, Japan) as a scintillation fluid, for the evaluation of uptake. Cellular protein content was determined by the BCA method (BCA Protein Assay Reagent Kit; Thermo Fisher Scientific, Waltham, MA), using bovine serum albumin as the standard. Uptake assays were also conducted in mock cells, which were transfected with empty pCI-neo vector, to estimate nonspecific uptake. The specific uptake of glycerol by AQP7 was estimated by subtracting its uptake in AQP7-expressing cells from that in mock cells.

Data analysis: The uptake of glycerol was evaluated at 5 min in the initial uptake phase, where uptake was in proportion to time,[5)] and the uptake rate (v) was calculated by dividing the uptake by time. The uptake clearance (CL_{up}) was calculated by dividing v by the substrate concentration (s). The saturable transport of glycerol in the absence of inhibitors was analyzed by assuming Michaelis-Menten type carrier-mediated transport, for which the CL_{up} is described as follows: $CL_{up} = V_{max}/(K_m + s)$. The kinetic parameters of maximum transport rate (V_{max}) and the Michaelis constant (K_m) were estimated by fitting this equation to the experimental data profile of CL_{up} *versus* s by using a nonlinear least-squares regression analysis program, WinNonlin (Pharsight, Mountain View, CA). The CL_{up} in the presence of a competitive inhibitor, of which the concentration and the inhibition constant are i and K_i, respectively, is as follows: $CL_{up} = V_{max}/(K_m(1 + i/K_i) + s)$. With the values of V_{max} and K_m fixed at those determined in the absence of inhibitors, K_i was estimated by fitting this equation to the profile of CL_{up} *versus* s for a given i.

Experiments were conducted in multiple preparations of cells and, in each preparation, the CL_{up} values were estimated as the means of duplicate determinations. A set of V_{max}, K_m and K_i were estimated in each preparation and then the means of parameters were estimated with their respective SE values, using multiple sets of parameters.

Results and Discussion

We first examined the effect of glycerol derivatives (**Supplementary Fig. 1**) on the specific uptake of glycerol (0.05 μM) by AQP7 at their varied concentrations. The concentration of glycerol was set to be much lower than the K_m for glycerol, which was 11.8 μM as the mean value estimated below in the present study, for the evaluation of their effects in the concentration range where glycerol transport is most efficient in the linear phase of transport kinetics and most sensitive to inhibitors. As shown in **Figure 1A**, AQP7-specific glycerol uptake was extensively inhibited by monoacetin, with almost complete inhibition being achieved at 1 mM. Inhibition by diacetin was also significant, although it was less extensive, with almost complete inhibition being achieved at 10 mM. Inhibition by triacetin was, however, insignificant even at the highest concentration of 10 mM. Thus, a decrease in the number of hydroxyl groups attached to the carbon backbone of the glycerol molecule by acetyl derivatization was associated with a decrease in inhibitory potency, suggesting a decrease in affinity for AQP7. Similarly, AQP7-specific glycerol uptake was extensively inhibited by monobutyrin, with almost complete inhibition being achieved at 1 mM (**Fig. 1B**). Although the effect of dibutyrin, which was not available, was not tested, the inhibitory effect of tributyrin was much weaker, being only modest with about 30% inhibition at its highest possible concentration of 500 μM. Thus, consistent with the results for acetyl derivatives, masking hydroxyl groups of glycerol by butyryl derivatization was also associated with a decrease in inhibitory potency. We also examined the effect

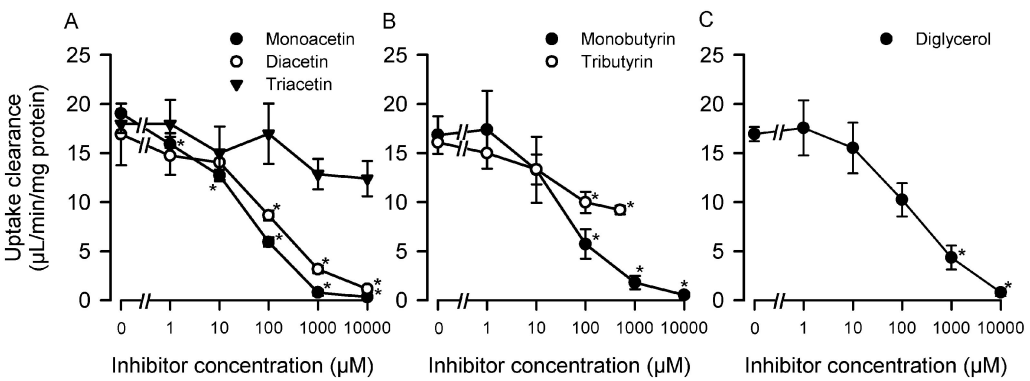

Fig. 1. Effect of various glycerol derivatives on glycerol uptake by AQP7 stably expressed in MDCKII cells
The specific uptake of [^{3}H]glycerol (0.05 μM) by AQP7 was evaluated for 5 min at 37°C and pH 7.4 in the presence of a glycerol derivative (inhibitor) at varied concentrations or in its absence. Data are presented as means ± SE ($n = 3$). * $p < 0.05$ compared with the control in the absence of inhibitors, as assessed using ANOVA followed by Dunnett's test.

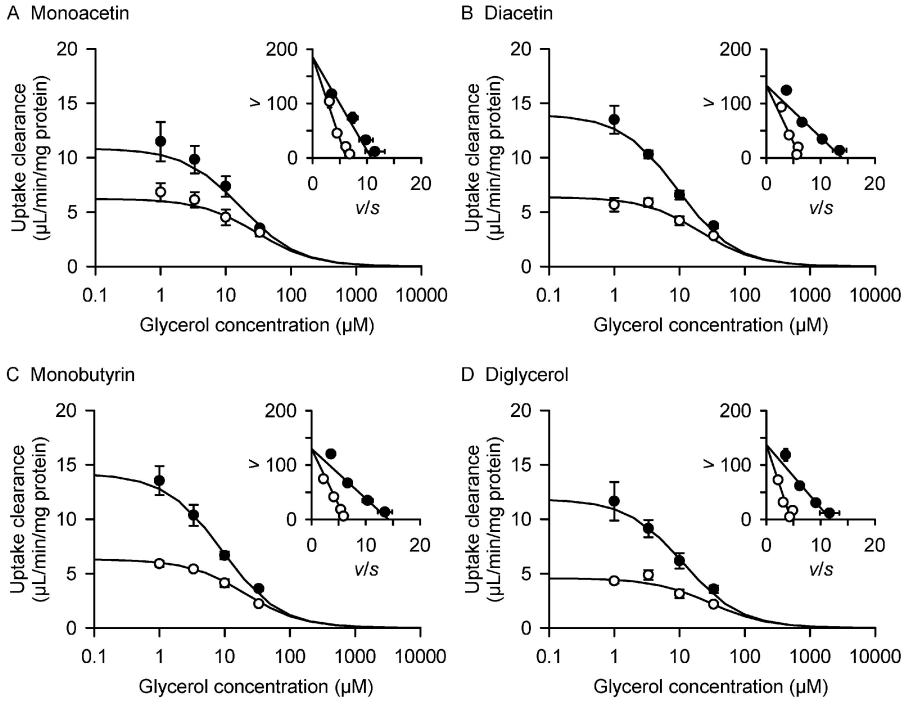

Fig. 2. Competitive inhibition of glycerol uptake by AQP7 stably expressed in MDCKII cells by various glycerol derivatives
The specific uptake of [^{3}H]glycerol by AQP7 was evaluated for 5 min at 37°C and pH 7.4 in the presence of a glycerol derivative (open circles) or in its absence (closed circles). The profiles of uptake clearance *versus* glycerol concentration were used for curve-fitting analyses for the estimation of kinetic parameters and Eadie-Hofstee plots are presented for graphical inspection. In the panels of A for monoacetin (100 μM), B for diacetin (500 μM), C for monobutyrin (100 μM) and D for diglycerol (500 μM), the values of V_{max} (pmol/min/mg protein) were 186 ± 26, 133 ± 20, 130 ± 9 and 137 ± 20, respectively, and those of K_m (μM) were 17.1 ± 5.0, 9.5 ± 2.4, 9.1 ± 1.6 and 11.6 ± 2.2, respectively. The values of K_i (μM) of monoacetin, diacetin, monobutyrin and diglycerol were 134 ± 17, 420 ± 111, 80 ± 18 and 312 ± 69, respectively. Data and parameters are presented as means ± SE ($n = 4$). The K_i values of monoacetin and monobutyrin were significantly different from that of diacetin at $p < 0.05$, as assessed using ANOVA followed by the Student-Newman-Keuls test. Differences between all the other pairs of K_i values were statistically insignificant.

of diglycerol, an ether type of glycerol derivative, on AQP7-specific glycerol uptake. Inhibition by this derivative was also significant and comparable with that by diacetin, with almost complete inhibition being achieved at 10 mM (**Fig. 1C**).

We then examined the effect of those glycerol derivatives on the kinetics of AQP7-mediated glycerol transport to probe into the inhibition mechanism. Triacetin and tributyrin were, however, excluded in this section because their inhibitory effects were insignificant or too weak for kinetic analyses. Kinetic analyses indicated that, as shown in **Figure 2**, all the glycerol derivatives tested inhibit AQP7-mediated glycerol transport in a manner conforming to the competitive model of Michaelis-Menten kinetics. Taking it into account that they all inhibited AQP7-specific glycerol uptake almost completely at higher concentrations (**Fig. 1**), it may be possible that they share the substrate recognition site with glycerol and are transported as substrates. The K_i of monoacetin (134 μM) was significantly smaller than that of diacetin (420 μM), but greater than the K_m for glycerol, which ranged from 9.1 to 17.1 μM as values determined in different sets of studies for various glycerol derivatives and was 11.8 μM as the mean value. It should also be noted that triacetin does not have an appreciable affinity, which leads to inhibitory effect, for AQP7, as indicated in **Figure 1**. Therefore, it is likely that a decrease in the number of hydroxyl groups in the glycerol molecule by acetyl derivatization leads to a decrease in affinity for AQP7, consistent with the indication of a decrease in inhibitory potency in the preceding section. The K_i of monobutyrin (80 μM) was, on the other hand, comparable with that of monoacetin, suggesting that the extension of the acyl chain by two hydrocarbon units does not have an impact on affinity for AQP7. The K_i of diglycerol (312 μM) was comparable with that for diacetin. Even though this ether type of glycerol derivative has four hydroxyl groups, its affinity for AQP7 was thus suggested not to be higher than those of acyl derivatives with fewer hydroxyl groups.

In conclusion, monoacetin, diacetin and monobutyrin, which are acyl glycerol derivatives, and also diglycerol, an ether type of glycerol derivative, were found to inhibit AQP7-mediated glycerol transport competitively. It may be possible, therefore, that they are all recognized by AQP7 as substrates competitive to glycerol. It is notable that a decrease in the number of hydroxyl groups by acetyl derivatization of glycerol was linked to a decrease in affinity for AQP7, indicating the important role of hydroxyl groups in recognition by AQP7. On the other hand, monoacetin and monobutyrin were comparable in affinity for AQP7, suggesting that the extension of the acyl chain by two hydrocarbon units does not have an impact on affinity for AQP7. Although it has already been suggested that hydroxyl groups in the glycerol molecule may play a role in its recognition by the glycerol facilitator of *Escherichia coli* (GlpF) as a bacterial ortholog of aquaglyceroporins,[8,9] this study is the first to suggest such a role of hydroxyl groups in glycerol recognition for a human aquaglyceroporin and to indicate additionally that the affinity of glycerol derivatives depends on the number of hydroxyl groups in their molecules. This study has also provided the first demonstration of competitive inhibitors for an aquaglyceroporin.

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