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Tolbutamide Uptake Via pH- and Membrane-potential-dependent Transport Mechanism in Mouse Brain Capillary Endothelial Cell Line

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Summary: The purpose of this study was to investigate the transport mechanism of tolbutamide across the blood-brain barrier (BBB) using MBEC4 cells as an in vitro BBB model.

Methods: The BBB transport of tolbutamide was studied by using a mouse brain capillary endothelial cell line, MBEC4, cultured on dishes with their luminal membrane facing the culture medium.

Results: The uptake of [14C]tolbutamide by MBEC4 cells was dependent on temperature and energy. The uptake coefficient of [14C]tolbutamide increased markedly with decreasing pH of the external medium from neutral to acidic. Valinomycin and replacement of chloride with sulfate or gluconate significantly increased the initial uptake of [14C]tolbutamide, while replacement with nitrate significantly decreased it. The uptake was significantly reduced by a proton ionophore, FCCP, and an anion-exchange inhibitor, DIDS. The initial uptake of [14C]tolbutamide was saturable with Kt of 0.61 ± 0.03 mM (pH 7.4) and 1.76 ± 0.19 mM (pH 6.5). At pH 6.5, the initial uptake of [14C]tolbutamide was significantly reduced by several sulfa drugs, salicylic acid, valproic acid and probenecid, and was competitively inhibited by sulfaphenazole (Ki = 3.47 ± 0.50 mM) and valproic acid (Ki = 2.29 ± 0.43 mM).

Conclusion: These observations indicate the existence of a pH- and membrane-potential-dependent anion exchange and/or proton-cotransport system(s) for concentrative uptake of tolbutamide and sulfa drugs in MBEC4 cells.

Key words: blood-brain barrier; brain capillary endothelial cells; organic anion

Introduction

The distribution of drugs to brain is mainly controlled by the blood-brain barrier (BBB), which is formed by brain capillary endothelial cells connected by tight junctions. Some drugs may pass through the BBB by passive diffusion in a lipophilicity-dependent manner, but the accumulation of some lipophilic drugs is prevented by active efflux systems such as P-glycoprotein in the BBB. On the other hand, some drugs, which have structures analogous to those of nutrients, are transported across the BBB via the transport systems for nutrients. There have been several in vivo and in vitro studies of the brain distribution of actively transported drugs, but BBB transport systems are less well known at the molecular level than those in other tissues and organs.

Sulfonylureas, tolbutamide and glibenclamide, are insulin secretagogues widely used to stimulate insulin secretion in the treatment of non-insulin dependent diabetes. Autoradiographic binding studies using

binding sites are primarily on neuronal cell bodies and on axon terminals, which mediate the release of neurotransmitters such as glutamate and GABA. However, there is no report concerning side effects of sulfonylureas in the brain. In fact, we have demonstrated that the distribution of tolbutamide into the brain was lower in comparison with that into other organs or tissues in rat. Furthermore, using a monolayer of mouse brain capillary endothelial cells, we showed that the transport of tolbutamide across the BBB is bidirectional (luminal-to-abluminal and abluminal-to-luminal) and both transports were saturable. It is conceivable that the bidirectional transport of tolbutamide across the BBB involves carrier-mediated transport. However, it is not yet known which transport system(s) are involved in the transport of tolbutamide across the luminal and abluminal membranes of brain capillary endothelial cells.

Organic anions require a transport system to transfer across cell membranes. Recently, several transport proteins for organic anions, such as MCT1, anion exchanger 2, AE2, OAT1, OAT2, OAT3, oatp1, oatp2, oatp3, and OAT-K1, have been isolated from liver, kidney and intestine. These transporters have been shown to transport various anionic drugs possessing a carboxyl or hydroxyl moiety, or conjugates with glucuronic acid, glutathione or sulfate, and so on. Both tolbutamide and sulfonamides possessing a sulfonyl moiety are acidic in nature. Their transport across cell membranes has not yet been investigated.

In this study, in order to further investigate the transport mechanism of tolbutamide across the BBB, we characterized the uptake transport of tolbutamide from the blood side into the brain endothelial cells using the MBEC4 cell line. It has been demonstrated that MBEC4 cells form a monolayer in a polarized fashion with their abluminal membrane facing the dish and their luminal membrane facing the culture medium. Since tolbutamide is an organic anion, we examined whether the uptake of tolbutamide is inhibited by the substrates of organic anion transporters, which are found in intestine, kidney, liver and so on.

Materials and Methods

Materials: [Ring-U-14C]Tolbutamide (2.18 GBq/mmol) and [2-14C]Diazepam (2.04 GBq/mmol) were purchased from Amersham Japan Ltd. (Tokyo, Japan). [Fructose-1-3H]Sucrose (455.1 GBq/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Tolbutamide, sodium salicylate, lactic acid, digoxin, sodium taurocholate and 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) were purchased from Nacalai Tesque (Kyoto, Japan). Sulfaphenazole, sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfisoxazole, sulfapyridine, sulfasalazine, sodium valproate, sodium L-aspartate, sodium estrone 3-sulfate and trihydroxymethylaminomethane (Tris) were purchased from Sigma (St. Louis, MO, USA). Carboxylycyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were commercial products of reagent grade.

Cell culture: Establishment of the MBEC4 cell line by infecting isolated mouse brain endothelial cells with SV40, and the biochemical and morphological characteristics of the cells have been described previously. The cells were maintained as previously described. Briefly, MBEC4 cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 µg/mL of streptomycin at 37°C in a 5% CO2-95% air atmosphere. MBEC4 cells were seeded at a density of 4 × 104 cells/mL on multidishes (Nunc, Denmark). The cells were grown for 3 days prior to transport experiments.

Uptake experiments: Uptake experiments with the cells on multidishes were run in the uptake buffer (141 mM NaCl, 4 mM KCl, 2.8 mM CaCl2, 1 mM MgSO4, 10 mM D-glucose and 10 mM 2-[4-(2-hydroxyethyl)pyridazinyl]ethanesulfonic acid (HEPES) for pH 7.4 or 10 mM 2-[N-morpholino]ethanesulfonic acid (Mes) for pH 6.5) at 37°C. MBEC4 cells grown on the multidish were washed three times with 1 mL of the uptake buffer (pH 7.4). The test solution (250 µL) (pH 7.4 or 6.5) was added to initiate the uptake and the cells were incubated at 37°C for the time indicated. To terminate uptake, the test solution was removed by suction and the cells were washed three times with 1 mL of the uptake buffer (pH 7.4 or 6.5). For the quantitation of the drug uptake, the cells were solubilized with 200 µL of 3 M NaOH followed by neutralization with HCl.

The amount of test compound taken up by the cells was estimated from the radioactivity in the sample and expressed as the uptake rate (nmol/time/mg protein), which was defined as the value obtained by dividing the uptake amount (nmol/mg protein) by the uptake time, as the cell-to-medium concentration (C/M) ratio (µL/mg protein), obtained by dividing the uptake amount by the initial concentration in the uptake buffer and...
correction for the cellular protein amount, or as the uptake coefficient (µL/time/mg protein), obtained by dividing the C/M ratio by the uptake time. Normalization of uptake by using the C/M ratio is convenient to assess and compare the uptake of different substrates at different concentrations. Radioactivity was determined using a liquid scintillation counter (LC6500, Beckman). Cellular protein was measured by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as the standard. Protein content of a multidish was 163.±±2.9 µg/well. [3H]Sucrose was used to estimate extracellular trapping amount. The uptake of [3H]Sucrose as the extracellular trapping amount at 1 min was 0.0629±0.0080 µL/mg protein (mean±S.E.M), which was considered to be negligible. Details of the conditions for each experiment are given in the figure legends or table footnotes.

Evaluation of transstimulation effect of unlabeled tolbutamide in MBEC4 cells depleted of ATP by azide treatment: ATP-depleted MBEC4 cells were prepared with glucose-depleted buffer containing 10 mM Na2SO4 for 10 min. These ATP-depleted cells were preloaded with the buffer containing or not containing 10 mM unlabeled tolbutamide for 10 min. After removal of the preincubation buffer by aspiration, the amount of [14C]tolbutamide taken up at 37°C was determined by incubating the cells in uptake buffer (pH 7.4), and measuring the radioactivity.

Evaluation of inhibitory effect of metabolic inhibitor, ionophore or transporter-inhibitors, and effect of replacement of sodium or chloride: MBEC4 cells were preincubated with sodium azide, 2,4-dinitrophenol (2,4-DNP), FCCP, amiloride, DIDS or valinomycin for 10 min. The effects of amiloride or valinomycin were measured in uptake buffer containing 0.1% dimethyl sulfoxide (DMSO) or 0.1% ethanol. MBEC4 cells were preincubated with each buffer without sodium or chloride ion for 10 min. The effects of replacing sodium or chloride ion with another cation or anion were measured after pH adjustment with Tris/HEPES. After removal of the preincubation buffer by aspiration, the amount of [14C]tolbutamide taken up at 37°C for 1 min was determined by incubating the cells in uptake buffer (pH 7.4) with each reagent, and measuring the radioactivity.

Evaluation of inhibitory effect of sulfa drugs, unlabeled tolbutamide and other compounds: The amount of [14C]tolbutamide taken up at 37°C for 10 sec was determined by incubating MBEC4 cells in uptake buffer (pH 6.5) with each compound and measuring the radioactivity and expressed as the uptake coefficient (% of control), determined as the C/M ratio at 10 sec after subtraction of the nonspecific binding to the cellular surface, which was estimated from the intercept on the vertical axis of the typical time course at pH 6.5. Except for the data in Fig. 4 and 5, values were not corrected for nonspecific binding. Because of low solubility, inhibitor concentrations in the incubation medium were limited to 0.2–2 mM for sulfaphenazole, 0.2–5 mM for sulfamethoxazole, 0.2–1 mM for sulfadimethoxine, 0.2–4 mM for sulfamethazine, 0.2–3 mM sulfisoxazole, 0.2–1 mM sulfa pyridine, 0.1–0.5 mM for sulfasalazine, 0.2–4 mM for tolbutamide and 0.001–0.1 mM for digoxin.

Data analysis: In order to estimate the kinetic parameters of the saturable transport across MBEC4 cells, the uptake rate (J) was fitted to the following equation which contains both saturable and non-saturable linear terms, using the program MULTI.17

\[ J = \frac{J_{max}}{K_t + S} + kd \times S \]

where \( J_{max} \) is the maximum transport rate for the carrier-mediated process, \( S \) is the concentration of the substrate, \( K_t \) is the half-saturation concentration, and \( kd \) is the first-order rate constant. All of the data were expressed as mean±S.E.M. and statistical analysis was performed by using Student’s two-tailed t-test or ANOVA followed by Dunnett’s test. A difference between means was considered to be significant when the P value was less than 0.05.

Results

Time course and temperature dependence of [14C]tolbutamide uptake by MBEC4 cells: The C/M ratio of [14C]tolbutamide increased time-dependently at 37°C, and reached a plateau of around 20 µL/mg protein at 1 min (Fig. 1A, 1B). About 20–40% of [14C]tolbutamide uptake at 1 min at 37°C was considered to represent adsorption on the cellular surface, as judged from the intercept on the vertical axis. The C/M ratio was significantly reduced at 4°C (Fig. 1A).

Effect of Triton-X 100 on the uptake of [14C]tolbutamide in MBEC4 cells: To determine whether the apparent concentrative uptake might simply represent passive entry followed by intracellular binding, we tested the effect of cell membrane permeabilization on tolbutamide uptake by MBEC4 cells (Fig. 1B). The C/M ratio of [14C]tolbutamide was significantly reduced by pretreatment of the cells with Triton-X 100 (0.015%). However, the C/M ratio was scarcely decreased by pretreatment with Triton-X 100 in the presence of 10 mM sodium azide to block the function of the putative active transporter of tolbutamide (Fig. 1A). On the other hand, the initial C/M ratio of [3H]diazepam was slightly increased by Triton-X 100 pretreatment, and similar steady-state values were obtained with and without the pretreatment (Fig. 1C).

Transstimulation effect of unlabeled tolbutamide on [14C]tolbutamide uptake by MBEC4 cells: To establish whether carrier-mediated transport was involved in
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tolbutamide uptake by MBEC4 cells, we tested the transstimulation effect. Figure 2 shows the uptake of \([14C]\)tolbutamide by MBEC4 cells, ATP-depleted in the presence of 10 mM unlabeled tolbutamide for 10 min. The cells preloaded with unlabeled tolbutamide showed a significantly enhanced uptake of \([14C]\)tolbutamide as compared to the cells preloaded with buffer.

Effects of metabolic inhibitor, pH, and a protonophore or a sodium/proton antiport inhibitor on \([14C]\)tolbutamide uptake by MBEC4 cells: The effects of metabolic inhibitor, pH, and a protonophore or a sodium/proton antiport inhibitor on the uptake of \([14C]\)tolbutamide were studied to determine whether this carrier-mediated transport requires metabolic energy and is driven by an inwardly directed proton or sodium gradient (Table 1 and Fig. 3). Sodium azide (10 mM) or 2,4-DNP (1 mM), metabolic inhibitors significantly diminished the uptake of \([14C]\)tolbutamide (Table 1).

The uptake coefficient of \([14C]\)tolbutamide increased markedly with decreasing pH of the external medium from neutral to acidic (Fig. 3). In the presence of 500 μM unlabeled tolbutamide, the uptake of \([14C]\)tolbutamide was significantly reduced at every pH examined, and the inhibitory effect was greater at acidic pH. Furthermore, the uptake of \([14C]\)tolbutamide was significantly reduced in the presence of a metabolic inhibitor, 10 mM sodium azide, as shown in Fig. 1. The decrease in the uptake coefficient of \([14C]\)tolbutamide by unlabeled tolbutamide and metabolic inhibitor was greater at acidic pH. To determine whether the uptake of tolbutamide by MBEC4 cells is dependent on the external pH or an inwardly directed proton gradient, we tested the effect of a protonophore, FCCP, and a sodium/proton antiport inhibitor, amiloride, on the uptake of \([14C]\)tolbutamide by MBEC4 cells (Table 1). FCCP and amiloride significantly reduced the uptake. When sodium ion was replaced with choline or N-methylglucamine in order to investigate the involvement
of sodium ion, there was no significant change of initial uptake of \([^{14}C]\)tolbutamide by MBEC4 cells (Table 1).

Effect of anion-replacement, anion-exchanger inhibitor and membrane potential on \([^{14}C]\)tolbutamide uptake by MBEC4 cells: We next investigated the uptake of \([^{14}C]\)tolbutamide when chloride in the medium was replaced with nitrate, sulfate, or glucose in MBEC4 cells (Table 1). Replacement of chloride with sulfate or glucose significantly increased the initial uptake of \([^{14}C]\)tolbutamide, while replacement with nitrate significantly reduced it. The anion-exchange inhibitor, DIDS, also significantly reduced the initial uptake in a dose-dependent manner, whereas treatment with valinomycin significantly increased it. These results indicate the involvement of membrane potential-dependent transport in the tolbutamide uptake.

Effect of several inhibitors on \([^{14}C]\)tolbutamide uptake by MBEC4 cells: To study the specificity of the carrier responsible for the uptake of tolbutamide by MBEC4 cells, we investigated the effects of sulfa drugs on the initial uptake of \([^{14}C]\)tolbutamide. The uptake of \([^{14}C]\)tolbutamide uptake by MBEC4 cells was significantly reduced by sulfa drugs such as sulfaphenazole, sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfisoxazole, sulfapyridine, and sulfasalazine (Fig. 4). We also investigated the inhibitory effect of various anionic compounds reported to be taken up via carrier-mediated transport on the \([^{14}C]\)tolbutamide uptake by MBEC4 cells. As shown in Fig. 5, there was no significant change of initial uptake of \([^{14}C]\)tolbutamide in the presence of 0.05–5 mM L-aspartic acid and L-glutamic acid, anionic amino acids, p-aminohippuric acid (a substrate of OAT1, OAT2 and OAT3),\(^{8–10}\) taurocholate (a substrate of the oatp family), estrone-3-sulfate (a substrate of the oatp family), and DMSO or ethanol (a specific inhibitor of organic cation transporter).\(^{18}\) Furthermore, 0.05–5 mM sulfate (an inorganic acid) also did not inhibit the initial uptake of \([^{14}C]\)tolbutamide by MBEC4 cells. On the other hand, the initial uptake was significantly decreased by salicylic acid and valproic acid, substrates of the monocarboxylate transporter, probenecid and sulfapyrazine, inhibitors of various anion transporters. The order of inhibitory potency of salicylazoxide > sulfonazoxide > valproic acid > probenecid > sulfamethoxazole > salicylic acid > sulfapyrazine.

Table 1. Inhibitory effect of metabolic inhibitors, ionophores, transporter-inhibitors, or sodium or chloride replacements on \([^{14}C]\)tolbutamide uptake

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration</th>
<th>C/M Ratio* (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN3 (-glucose)</td>
<td>10 mM</td>
<td>20.7±3.5**</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>1 mM</td>
<td>22.2±3.7**</td>
</tr>
<tr>
<td>FCCP</td>
<td>1 μM</td>
<td>39.0±4.0**</td>
</tr>
<tr>
<td>DIDS</td>
<td>0.5 mM</td>
<td>55.7±2.3***</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>1 mM</td>
<td>56.8±0.4**</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>0.05 mM</td>
<td>56.4±0.5***</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 mM</td>
<td>36.6±0.6**</td>
</tr>
</tbody>
</table>

Note: a MBEC4 cells were preincubated with NaN3 or 2,4-DNP for 10 min. No significant change of initial uptake of \([^{14}C]\)tolbutamide when chloride in the medium was replaced with nitrate, sulfate, or glucose (Table 1). Replacement of chloride with sulfate or glucose significantly increased the initial uptake of \([^{14}C]\)tolbutamide, while replacement with nitrate significantly reduced it. The anion-exchange inhibitor, DIDS, also significantly reduced the initial uptake in a dose-dependent manner, whereas treatment with valinomycin significantly increased it. These results indicate the involvement of membrane potential-dependent transport in the tolbutamide uptake.

Effect of several inhibitors on \([^{14}C]\)tolbutamide uptake by MBEC4 cells: To study the specificity of the carrier responsible for the uptake of tolbutamide by MBEC4 cells, we investigated the effects of sulfa drugs on the initial uptake of \([^{14}C]\)tolbutamide. The uptake of \([^{14}C]\)tolbutamide uptake by MBEC4 cells was significantly reduced by sulfa drugs such as sulfaphenazole, sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfisoxazole, sulfapyridine, and sulfasalazine (Fig. 4). We also investigated the inhibitory effect of various anionic compounds reported to be taken up via carrier-mediated transport on the \([^{14}C]\)tolbutamide uptake by MBEC4 cells. As shown in Fig. 5, there was no significant change of initial uptake of \([^{14}C]\)tolbutamide in the presence of 0.05–5 mM L-aspartic acid and L-glutamic acid, anionic amino acids, p-aminohippuric acid (a substrate of OAT1, OAT2 and OAT3),\(^{8–10}\) taurocholate (a substrate of the oatp family), estrone-3-sulfate (a substrate of the oatp family), and DMSO or ethanol (a specific inhibitor of organic cation transporter).\(^{18}\) Furthermore, 0.05–5 mM sulfate (an inorganic acid) also did not inhibit the initial uptake of \([^{14}C]\)tolbutamide by MBEC4 cells. On the other hand, the initial uptake was significantly decreased by salicylic acid and valproic acid, substrates of the monocarboxylate transporter, probenecid and sulfapyrazine, inhibitors of various anion transporters. The order of inhibitory potency of salicylazoxide > sulfonazoxide > valproic acid > probenecid > sulfamethoxazole > salicylic acid > sulfapyrazine.

Concentration dependence of tolbutamide uptake by
Inhibitory effects of sulfa drugs and unlabeled tolbutamide on [14C]tolbutamide uptake. The amount of [14C]tolbutamide taken up was measured at 37°C for 10 sec by incubating MBEC4 cells in uptake buffer (pH 6.5) with each drug. Control value after subtraction of nonspecific binding was 29.4 ± 0.85 (μL/10 sec/mg protein). Each value represents the mean ± S.E.M. of four experiments.

MBEC4 cells and analysis of the inhibitory mechanisms of sulfaphenazole and valproic acid: We examined the concentration dependence of tolbutamide uptake by MBEC4 cells at pH 7.4 and 6.5 (Fig. 6). The kinetic parameters obtained for tolbutamide uptake at pH 7.4 were Jmax 1.82 ± 0.06 nmol/10 sec/mg protein, Kt 0.61 ± 0.03 mM, and kd 3.19 ± 0.08 μL/10 sec/mg protein. Those at pH 6.5 were Jmax 57.7 ± 5.20 nmol/10 sec/mg protein, Kt 1.76 ± 0.19 mM, and kd 4.33 ± 0.44 μL/10 sec/mg protein. To study the mechanism of the inhibition by sulfaphenazole and valproic acid, the inhibitory effects at pH 6.5 were kinetically analyzed. Figures 7A and 7B show Eadie-Hofstee plots of the effects of sulfaphenazole and valproic acid, respectively, on the uptake of tolbutamide, indicating that sulfaphenazole and valproic acid are competitive inhibitors of tolbutamide uptake. The inhibitory constants (Ki) of sulfaphenazole and valproic acid at pH 6.5 were 3.47 ± 0.50 mM and 2.29 ± 0.43 mM, respectively.

Discussion

We have already reported that tolbutamide, which is frequently used in the treatment of diabetic patients, is exported from the brain to blood via a non-P-gp-eflux transport system which is inhibited by sulfonamides such as sulfaphenazole, and we suggested that this specific transport system may be responsible for the limited distribution of tolbutamide to the brain. However, it is not yet known which transporter(s) are involved in the transport of tolbutamide across the luminal and abluminal membranes at the BBB. In this study, we investigated the uptake mechanism of tolbutamide through the luminal membrane of brain capillary endothelial cells, MBEC4, cultured on dishes with their luminal membrane facing the culture medium. The C/M ratio of [14C]tolbutamide increased time-dependently, and reached a plateau at around 10–20 μL/mg protein at 1 min. This represents a 3–7 fold
Fig. 5. Inhibitory effects of various anions and cations on [14C]tolbutamide uptake. The amount of [14C]tolbutamide taken up was measured at 37°C for 10 sec by incubating MBEC4 cells in uptake buffer (pH 6.5) with each drug. Control value after subtraction of nonspecific binding was 25.6 ± 1.22 (μL/10 sec/mg protein). Each value represents the mean ± S.E.M. of four experiments.

Increase in concentration, based on a cellular volume of around 3 μL/mg protein (calculated from the steady-state uptake of 3-O-methylglucose; unpublished data). To rule out the possibility that the apparent concentration uptake might simply represent passive entry followed intracellular binding, we tested the effect of permeabilizing the cell membrane by pretreatment with Triton-X 100 (Fig. 1). We considered two possibilities. One is an increase in the C/M ratio if passive tolbutamide entry by diffusion were the rate-limiting step.
in gaining access to intracellular binding site(s). The other is a decrease in the C/M ratio, by introducing a leak pathway, if a membrane transporter were actively accumulating tolbutamide against a concentration gradient.\(^{19}\) The C/M ratio of \(^{14}\text{C}\)tolbutamide was significantly reduced by Triton-X 100, whereas Triton-X 100 had little effect on the ratio in cells which had been treated with 10 mM sodium azide to block the function of the putative active transporter. From this finding, we concluded that the concentrative accumulation of tolbutamide is not due to intracellular binding. For comparison, we investigated the effect of membrane permeabilization on uptake of diazepam, which shows apparently concentrative uptake by intracellular binding after passive diffusion into the cell.\(^{20}\) As expected, the initial C/M ratio of \(^{3}\text{H}\)diazepam was slightly increased by pretreatment with Triton-X 100, and showed similar steady-state values with or without the Triton-X 100 pretreatment. Furthermore, tolbutamide uptake showed a transstimulation effect (Fig. 2), and the uptake of \(^{14}\text{C}\)tolbutamide was decreased by treatment with a metabolic inhibitor, sodium azide (10 mM) or 2,4-DNP (1 mM) (Table 1). These results confirm the existence of an active transport system for concentrative tolbutamide uptake by MBEC4 cells.

What is the driving force for this active transporter of tolbutamide in MBEC4 cells? The uptake of many organic compounds, such as glucose and amino acids,
by mammalian cells is sodium ion-dependent but this was not the case for the initial uptake of \(^{14}\)C-tolbutamide by MBEC4 cells, because there was no significant change of tolbutamide uptake when sodium was replaced with other cations (Table 1). Another possible driving force in mammalian cells is a proton gradient.\(^{26}\) Because the sodium/proton antiporter is located in the luminal membrane of the brain capillary endothelial cells,\(^{29}\) an acidic microclimate pH exists at the surface of the luminal membrane and generates a proton gradient across the cell membrane. Indeed, the uptake coefficient of \(^{14}\)C-tolbutamide increased markedly with decreasing pH of the external medium to acidic from neutral (Fig. 3). Kinetic analysis showed that the activity of carrier-mediated transport at pH 6.5, expressed as Jmax/Km, was 32.8 \(\mu L/10 \text{ sec/mg protein}\), being 11-fold larger than that (3.00 \(\mu L/10 \text{ sec/mg protein}\)) at pH 7.4. In order to determine whether the uptake is dependent on the external pH or an inwardly directed proton gradient, we tested the effect of a protonophore, FCCP, and a sodium/proton antiport inhibitor, amiloride (Table 1). FCCP and amiloride both significantly reduced the uptake of \(^{14}\)C-tolbutamide. However, the sodium/proton antiporter may not be involved in the uptake as a source of supply driving force, because, as mentioned above, replacement of sodium with other cations had no effect. Therefore the effect of amiloride might not be due to the inhibition of a sodium/proton antiporter.

We also examined the effects of chloride replacement with other anions and membrane potential on the initial uptake of \(^{14}\)C-tolbutamide (Table 1). The uptake was significantly increased by replacement of chloride with sulfate or gluconate, but significantly reduced by replacement with nitrate. The permeation of sulfate or gluconate through the membrane is slower than that of nitrate or chloride, and therefore inside-negativity blocked tolbutamide transport. The uptake was significantly increased by valinomycin, a membrane potential disruptor (Table 1). Therefore, \(^{14}\)C-tolbutamide uptake was increased by the disruption of the normal negative membrane potential. Moreover, the initial uptake of \(^{14}\)C-tolbutamide was significantly reduced by the anion exchange inhibitor, DIDS (Table 1). These results indicated the involvement of a membrane potential-dependent transporter which exchanges tolbutamide for anions and/or exhibits proton-cotransport. A membrane vesicle study will be needed to analyze the driving force for this transporter.

Previously, we developed a physiological pharmacokinetic model to study the effect of various sulfonamides on the plasma elimination and brain distribution of \(^{14}\)C-tolbutamide in rats.\(^{22}\) Therefore, we investigated the structural specificity of the carrier responsible for tolbutamide initial uptake by MBEC4 cells. The initial uptake of \(^{14}\)C-tolbutamide was significantly reduced by sulfa drugs such as sulfaphenazole, sulfamethoxazole, sulfadimethoxine, sulfamethazine, sulfisoxazole, sulfapyridine and sulfosalazine in a concentration-dependent manner (Fig. 4). Because HEPES and MES contained in the uptake buffer are sulfonic acid derivatives, they may inhibit the transport of tolbutamide; this remains to be examined. Furthermore, sulfaphenazole competitively inhibited the uptake of tolbutamide, with a Ki value of 3.47 ± 0.50 mM (Fig. 7). Other possible substrates of carrier-mediated transport were also examined. As shown in Fig. 5, the initial uptake of \(^{14}\)C-tolbutamide was not affected by aspartic acid, glutamic acid, p-aminohippuric acid, bile acid, estrone-3-sulfate, digoxin, methotrexate or choline, indicating that anionic amino acid transporter,\(^{27}\) the organic anion transporter (OAT) family,\(^{2,28}\) the organic anion transporting polypeptide (oatp) family\(^{11,14}\) and organic cation transporter\(^{29}\) contribute little to the uptake of tolbutamide by MBEC4 cells. Furthermore, sulfate, which was reported to be transported across the plasma membrane by a sulfate transporter,\(^{30,31}\) did not inhibit the initial uptake of \(^{14}\)C-tolbutamide. The uptake was significantly decreased by salicylic acid, valproic acid and L-lactic acid, which are transported by monocarboxylate transporters,\(^{32}\) such as monocarboxylate/proton cotransporter (MCT)\(^{6}\) and anion exchanger 2 (AE2).\(^{33}\) Furthermore, valproic acid competitively inhibited the uptake of tolbutamide, with a Ki value of 2.29 ± 0.43 mM (Fig. 7B). These monocarboxylate transporters are candidates for the transporter of tolbutamide. If an electroneutral transporter such as MCT1 transported tolbutamide, some other electrogenic ion transport system would have to be involved in the rate-limiting step of the tolbutamide transport process.

We observed some variability in uptake activities; e.g., uptake for 10 sec at pH 7.4 in Fig. 3 is lower than that in Fig. 1B and uptake for 10 sec at pH 6.5 in Fig. 3 is lower than those in Fig. 4 – 7. One of the reasons for this difference might be differences in the passage number of cells, but these differences do not affect the overall conclusions.

Tolbutamide is used in the treatment of diabetic patients. Therefore the fact that it is a substrate for active bidirectional transport across the BBB raises the possibility of interaction with other drugs. Although there is no report of CNS toxicity of tolbutamide itself, it might modify the pharmacokinetics of other actively transported drugs with potentially toxic consequences. Further studies on the influx and efflux processes of tolbutamide at the abluminal and luminal membranes are desirable.

In conclusion, our results indicate the existence of a pH- and membrane-potential-dependent anion exchange and/or proton cotransport system(s) for...
concentrative uptake of tolbutamide and sulfa drugs in brain endothelial cells.

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