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Blood–Brain Barrier Controls Carnitine Level in the Brain: A Study on a Model System with RBE4 Cells

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Transport of carnitine was studied with immortalized rat brain endothelial cells (RBE4), an *in vitro* model of the blood–brain barrier. The experiments on uptake and efflux through the luminal membrane excluded any involvement of choline and amino acids transporters, as well as that of glycoprotein P. Acetyl-, octanoylcarnitine, and betaine were without any effect; the only compound decreasing both processes was butyrobetaine. An exposure of the abluminal membrane resulted in a 40% inhibition of carnitine uptake by the substrates of neutral amino acid transporter L, while its efflux through the basolateral membrane, occurring in a form of free carnitine, was sensitive to SH group reagent, mersalyl, and was diminished by butyrobetaine. These features of carnitine transport did not fully correspond to the known characteristics of the proteins transporting carnitine in other tissues (OCTN2 and CT1); however, they did not exclude an involvement of a transporter belonging to the same superfamily. Moreover, such a protein in brain endothelium would fulfill a regulatory role in the transport of carnitine through the blood–brain barrier.

The brain capillary endothelial cells connected by tight junctions provide a nonpermeable barrier for ions and polarized molecules between blood and brain fluids, the so-called blood–brain barrier. The accumulation of any compound in the brain depends on the selectivity of these cells, a phenomenon ascribed to the presence of several specialized transporting systems (1). One of the substances which accumulates in the brain is carnitine (4-trimethylamino-3-hydroxybutyrate). This compound, on the contrary to peripheral tissues where it is engaged in a transfer of acyl moieties into mitochondria for the process of β-oxidation, in case of cerebral cortical neurons was reported to fulfill a different role, namely an export of acetyl moiety from mitochondria to cytoplasm, thus promoting a synthesis of acetylcholine (2, 3). As reported elsewhere, carnitine accumulates in brain capillary endothelial cells (4, 5); moreover, an asymmetry of carnitine uptake was reported in case of a primary culture of porcine cells.

Recently, cDNA encoding a carnitine transporter (CT1) was isolated from rat intestine (6). Carnitine transport, measured after expression of CT1 in *Xenopus* oocytes, was observed to be inhibited by short- and medium-chain acylcarnitines, as well as by betaine and butyrobetaine (6). Since the last compound was also observed to decrease carnitine uptake through the luminal membrane of the porcine capillary endothelial cells (5), the present study was focused on the possible influence of the compounds affecting the activity of CT1 on the transport of carnitine in a pure line of immortalized rat brain endothelial cells, RBE4 (7). Moreover, since carnitine is known to be a substrate of carnitine acyltransferases (8), revealing their activity also in RBE4 cells (4), the experiments were carried out to define the form of carnitine released through the abluminal membrane, in order estimate a possible regulatory role of the blood–brain barrier in carnitine transport to the brain.

MATERIALS AND METHODS

Materials. RBE4 cells from rat brain microvessel endothelial cells were immortalized by transfection with the plasmid pE1A-*neo*, containing the adenovirus E1A gene, as described in (7). Tissue plastics were from Corning. Minimum essential medium (Alpha medium), Ham's F-10 nutrient mixture, fetal bovine serum, L-glutamine, gentamycin, geneticin, and collagen G were provided by Gibco, basic fibroblast growth factor by Boehringer. L-[Methyl-^{3}H]carnitine was purchased from Amersham. L-acylcarnitines were delivered by Serva, L-carnitine was provided by Fluka. Butyrobetaine ((3-carboxypropyl)trimethyl-ammonium) was synthesized by methylation of γ-aminobutyric acid by Dr. J. Boksa in the Institute of

Abbreviations used: BCH, 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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Pharmacology, Cracow, Poland. Aluminium silica gel 60 (sheets) was from Merck. Ultima Gold (Packard) was used as a scintillation cocktail. All other reagents were from Sigma.

Accumulation of carnitine. RBE4 cells were grown to confluence at 37°C in a humid atmosphere of 5% CO_2 in the medium described in (4). For the uptake experiments the culture medium was removed and cells were washed with phosphate-buffered saline (PBS). The cells were subsequently incubated at 37°C as a monolayer in PBS supplemented with 5 mM glucose. The reaction was started by addition of 50 μM L-[methyl-^{14}C]carnitine (15 Ci/mol) and terminated by removal of the incubation medium. Subsequently, the monolayer was washed three times with ice cold PBS, the cells were collected in PBS with a cell scraper, and spun down at 5,800 g for 10 min at 4°C. For the experiments performed with cells in suspension, the cellular monolayer was collected with a cell scraper in PBS with 5 mM glucose before incubation. The reaction was started with radioactive carnitine and terminated by spinning down at 5,800 g for 10 min at 4°C, followed by three PBS washes and centrifugation. For measurement of the total carnitine accumulation, cells were dissolved by incubating overnight in 0.1 M NaOH, 2% Na_2CO_3, 1% sodium dodecyl sulfate (SDS) at 40°C and taken for radioactivity counting. The results were calculated as the amount of carnitine referred to the protein content (4, 5). The unspecific binding was subtracted as the "zero time uptake". For estimation of carnitine and its derivatives accumulated in cell monolayer, the reaction was performed in the same way, with the exception of carnitine specific radioactivity, increased to 150 Ci/mol. The cellular pellet after termination of the reaction was treated with 10% trichloroacetic acid and the further procedure of carnitine and its acyl derivatives separation was performed by thin layer chromatography according to (9).

Carnitine efflux. The measurements of carnitine efflux through the apical membrane followed the technique applied by Estrada *et al.* (10). The cells were treated as for the uptake experiments in a monolayer and incubated for 1 h in the presence of 50 μM L-[methyl-^{14}C]carnitine (150 Ci/mol). At the end of the incubation period, the 6-well plates were put on ice for 10 min, the incubation medium was subsequently removed, the cell monolayer was washed 3 times with cold PBS, and covered with PBS (1 ml per well) with the additions indicated in the figure legends. The plates were transferred to 37°C. Aliquots (100 μl) of the medium were taken at indicated times for radioactivity counting and immediately the same volume of PBS with the indicated compounds was added. For the measurements of carnitine efflux through the basolateral membrane the cells were grown on the filters (Transwell-Clear, 0.4 μM pore size) covered with collagen. The cells were incubated with the radioactive carnitine as in case of efflux experiments through the apical membrane, the filters were subsequently transferred to another wells with PBS containing additions indicated in the figure legends. Aliquots of the medium from the lower compartment were taken for radioactivity counting after indicated time and the sample volume was compensated with the same buffer. For determination of the carnitine form found in the lower compartment, its content was lyophilized and subjected to chromatographic analysis, as described by Huth *et al.* (9).

RESULTS

Carnitine accumulation in RBE4 cells was studied as a function in time. As presented in Fig. 1, this process takes place when the cells are grown in a monolayer (panels A and C). The exposure of basolateral membrane in case of suspended cells did not significantly increase the accumulation of carnitine (panels B and D). Betaine slightly decreased the process studied in cell monolayer, without affecting, however, the initial velocity (Fig. 1A). On the contrary, butyrobetaine revealed an inhibitory effect already at short times both, in the monolayer and suspension of RBE4 cells, reaching 40% inhibition at longer times (Figs. 1A and 1B). It was observed previously that certain neutral amino acids decreased the accumulation of carnitine in the primary culture of porcine capillary endothelial cells (5). Similarly, leucine, phenylalanine and 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid (BCH) decreased carnitine accumulation upon exposure of the basolateral membrane, without any effect on the cells grown in monolayer (Figs. 1C and 1D). A similar asymmetric sensitivity was observed when the influence of acylcarnitines was analyzed in more detail (Fig. 2). Short chain derivative (acetylcarnitine), as well as the medium chain acylcarnitine (octanoylcarnitine), did not affect the accumulation of carnitine in cell monolayer, whilst their inhibitory effect was detected in the suspension of RBE4 cells—a phenomenon observed, however, only after longer times (Fig. 2).

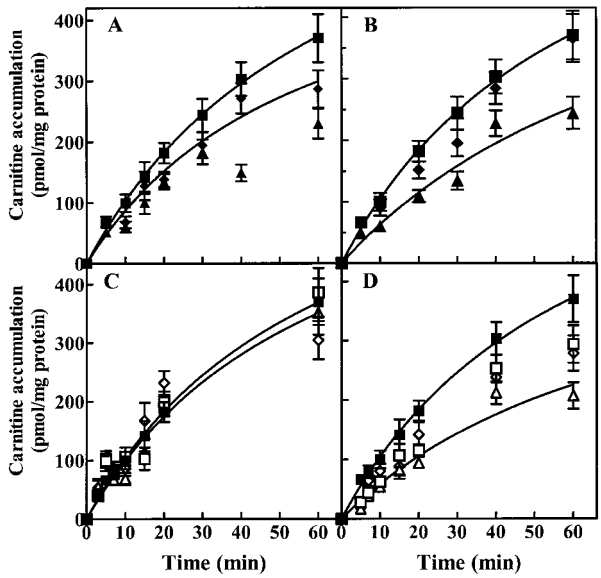

FIG. 1. Effect of betaines and amino acids on accumulation of carnitine in RBE4 cells. The uptake of 50 μM carnitine was measured either in cell monolayer (A, C) or in suspension of the cells (B, D), as described under Materials and Methods. The accumulation of carnitine was followed in the absence of any additions (squares) or upon simultaneous addition of the following compounds at 1 mM concentration: butyrobetaine (triangles), betaine (diamonds), phenylalanine (open diamonds), leucine (open triangles), BCH (open squares). The results are means ± SD from 3 independent experiments, in which the measurements were performed in triplicates.

Many compounds, when transported due to a facilitated diffusion, can be also transported outside the cells by the same transporting protein. As presented in Fig. 3, carnitine can be released by the RBE4 cells when this compound is missing in external medium. BCH did not influence the efflux of carnitine through the apical membrane (Fig. 3A), while an inhibitory effect of butyrobetaine was already observed at the

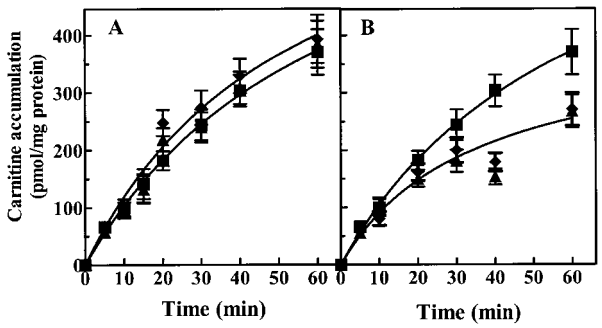

FIG. 2. Effect of acylcarnitines of various chain length on accumulation of carnitine in RBE4 cells. The uptake of 50 μM carnitine was measured either in cell monolayer (A) or in suspension of the cells (B), as described under Materials and Methods. The accumulation of carnitine was followed in the absence of any additions (squares) or in the presence of either 1 mM acetylcarnitine (triangles) or 1 mM octanoylcarnitine (diamonds). The results are means ± SD from 3 independent experiments, in which the measurements were performed in duplicates.

shortest times (Fig. 3B). Neither hemicholinium-3, a known inhibitor of choline transporter (Fig. 3C) (11), nor vincristine (Fig. 3D), inhibiting the glycoprotein P, known to remove from the cells many hydrophobic

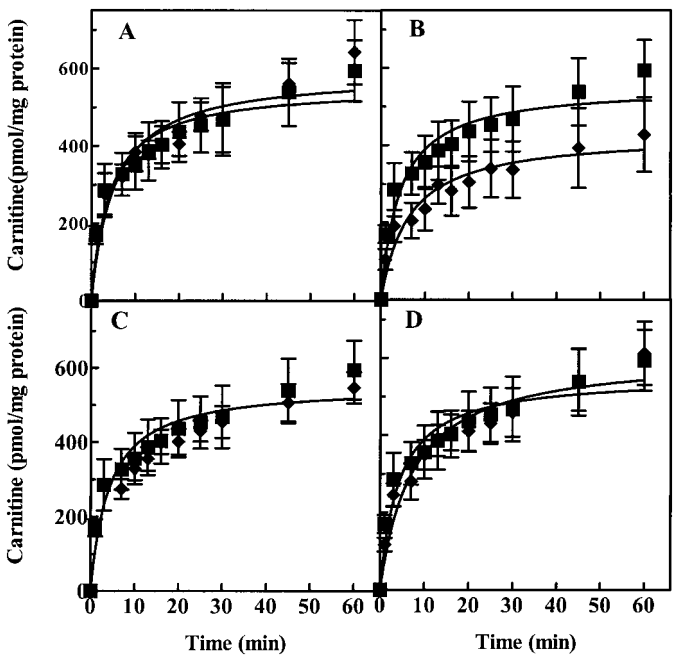

FIG. 3. Carnitine release from the RBE4 cells. The cells, grown in a monolayer, were loaded with 50 μM carnitine for 1 h and the efflux of radioactivity was subsequently followed as a function of time, as described in detail under Materials and Methods, according to Estrada *et al.* (10). The release was measured without any additions (squares) or upon addition of the following compounds (diamonds): 1 mM BCH (A), 1 mM butyrobetaine (B), 2 μM hemicholinium-3 (C), 50 μM vincristine (D). The results are means ± SD from 3 independent experiments, in which the measurements were performed in triplicates.

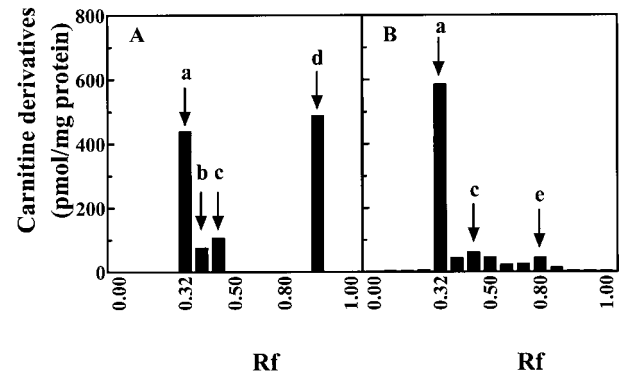

FIG. 4. Analysis of the forms of carnitine accumulated and released by RBE4 cells. RBE4 cells were incubated for 1 h with 50 μM carnitine, either in a monolayer (A) or grown on the filter inserts (B). Estimation of the amounts of carnitine and its derivatives was performed, as described under Materials and Methods, by thin-layer chromatography according to Huth *et al.* (9). (A) Represents the analysis of carnitine derivatives accumulated in the cells. (B) The analysis of medium below the filters after 1 h efflux. Arrows indicate the positions of following carnitine derivatives: a, free carnitine; b, acetylcarnitine; c, propionylcarnitine; d, long-chain acylcarnitines; e, laurylcarnitine.

compounds (12), affected the measured release of carnitine, thus excluding a possible involvement of these two proteins in the process of carnitine efflux through an apical membrane.

Carnitine, when accumulated in endothelial cells, becomes a substrate of several carnitine acyltransferases (8). In case of RBE4 cells it is accumulated mainly as free carnitine and the long-chain acylcarnitines, while the other forms of acylcarnitines did not exceed 25–30% (Fig. 4A). In order to define if carnitine can be released through the basolateral membrane, the cells were grown on the filter insert. An appearance of the radioactive compound in the lower compartment seemed to prove such a possibility. Moreover, a detailed chromatographic analysis (Fig. 4B) demonstrated that the main form found in the lower compartment is free carnitine, the low amounts of detected acetylcarnitine and laurylcarnitine did not exceed significantly the background detection of radioactivity. An attempt was further undertaken to define a system responsible for carnitine release through the basolateral membrane. The compounds expected to affect this process were added to a lower compartment, after a previous loading of the cells with radioactive carnitine. As presented in Fig. 5, the carnitine efflux was detected to be diminished in the presence of butyrobetaine (Fig. 5A) and an SH group regent, mersalyl (Fig. 5B).

DISCUSSION

Brain microcapillary endothelial cells accumulate carnitine mainly through the apical membrane, since

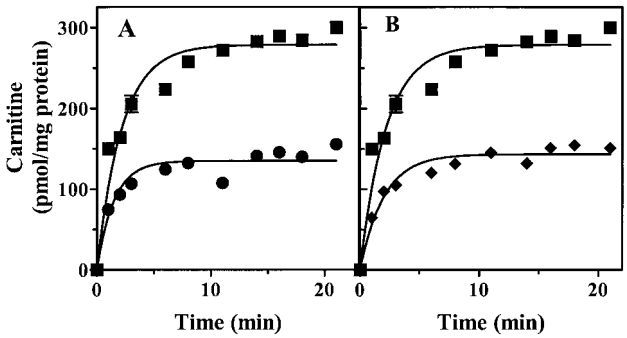

FIG. 5. Efflux of carnitine through the basolateral membrane of RBE4 cells. The cells were grown on filter inserts and loaded for 1 h with 50 μM carnitine, as described under Materials and Methods. The filters were subsequently transferred to another compartments and the appearance of carnitine in the lower compartments was followed. The measurements were performed in the absence (squares) or presence of the following additions: 1 mM butyrobetaine (circles), 0.1 mM mersalyl (diamonds). The results are means ± SD from 3 independent experiments, in which the measurements were performed in triplicates.

the exposure of the basolateral membrane after suspending the cells did not increase dramatically the total uptake. Due to the fact that carnitine transport through the basolateral membrane was observed to be decreased by leucine, phenylalanine and BCH, an involvement of amino acid transporter, specific toward neutral amino acids (13), could be postulated, although, it is worth emphasizing, that this particular process of carnitine accumulation most probably does not have any physiological meaning. On the contrary, the process of carnitine transport through the apical membrane seems to be more specific, since, out of many compounds studied, only γ-butyrobetaine inhibited the accumulation of carnitine. A lack of any effect of choline and γ-aminobutyric acid (not shown) would lead to the conclusion that the carboxyl- and trimethylamino-groups are more important for the transport phenomenon than the hydroxyl one. It seems, as well, that the length of carboxyl chain is a crucial parameter of the transported compound; betaine, shorter from butyrobetaine by two carbons, did not reveal any effect on carnitine accumulation.

Carnitine transport to various tissues has been characterized functionally, the common features seem to be Na-dependence and inhibition by several compounds, including D-carnitine, γ-butyrobetaine, acetylcarnitine (9, 14–22). Although the accumulation of carnitine in brain microcapillary endothelial cells was observed to be sensitive to ouabain, only when the cells were in suspension (4, 5), it can not be excluded, however, that the Na^+, K^+-ATPase present in the apical membrane is less sensitive to ouabain, a phenomenon ascribed to the presence of α_1 isoform of this subunit of the enzyme (23, 24). Moreover, an asymmetric localization of the Na^+, K^+-ATPase in the blood–brain barrier has been observed (25). Sodium dependence of carnitine transport was also reported after expression in *Xenopus laevis* oocytes of size-fractionated rat renal–cortex mRNA a process observed to be strongly inhibited by acetylcarnitine and butyrobetaine (26). Recently, the sequences of two proteins capable of transporting carnitine were obtained by molecular cloning. One of them, OCTN2, belongs to the family of sodium ion-dependent organic cation transporters (27), the other one, carnitine transporter (CT1) from rat intestine, after phylogenetic analysis, seems to be located in the midpoint between organic anion transporters and organic cation transporters (6). Carnitine transport catalyzed by both of these proteins was not only inhibited by butyrobetaine, but also strongly diminished by acetylcarnitine (6, 27), moreover, the activity of CT1 was reported to be strongly decreased by betaine (28). Our observations, demonstrating a lack of any effect of short- and medium-chain acylcarnitines and betaine on carnitine accumulation in the brain capillary endothelial cells. It would indicate an involvement of a protein different from OCTN2 and CT1, a suggestion supported by a very low expression of these proteins in brain (27, 28). The sequence analysis of OCTN2 resulted in finding of a nucleotide binding motif similar to the one of ABC transporters (27). However, the inhibitor of the glycoprotein P, belonging to the ABC transporters, did not affect carnitine release. Taking all this together, the presence in brain endothelium of a protein not identical to OCTN2 and CT1, but belonging to the same multispecific organic ion transporter superfamily (6, 29) may be postulated, especially that our attempts to purify a protein bound to activated thiol-Sepharose resin and subsequently eluted with carnitine, resulted in obtaining of a polypeptide of about 50 kDa (not shown), i.e., similar to the molecular weight of organic ion transporters. It seems that the protein responsible for the transport of carnitine through the blood–brain barrier is characterized by a rather strict substrate specificity. This, and the fact that carnitine crosses the monolayer of endothelial cells and is released in its free form, points to the putative carnitine transporter studied herewith as responsible for the regulation of carnitine accessibility to the brain.

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