Carrier-Mediated Transport of H_1-Antagonist at the Blood–Brain Barrier: Mepyramine Uptake into Bovine Brain Capillary Endothelial Cells in Primary Monolayer Cultures

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The transport mechanism of the H_1-antagonist mepyramine at the blood-brain barrier (BBB) was studied by using primary cultured monolayers of bovine brain capillary endothelial cells (BCEC). The initial uptake of [^{3}H]mepyramine into the BCEC showed strong temperature and concentration dependency, indicating that it involves both saturable and nonsaturable processes. Transport at the luminal membrane may be the rate-limiting process in the transcellular transport, since the values of the uptake coefficient of [^{3}H]mepyramine at the luminal membrane (609 μl/mg protein/min) and the transcellular permeability coefficient (488 μl/mg protein/min) are very similar. The initial uptake of [^{3}H]mepyramine was not affected by metabolic inhibitors, but was stimulated by preloading with the drug. Mepyramine appears to be transported into the BCEC by a carrier-mediated transport system which does not require metabolic energy, probably via a facilitated diffusion mechanism.

KEY WORDS: mepyramine; H_1-antagonist; blood-brain barrier transport; carrier-mediated transport; primary cultured brain capillary endothelial cells.

INTRODUCTION

Classical H_1-antagonists such as chlorpheniramine, diphenhydramine and diphenylpyraline have been used for the treatment of allergic disorders for a long time. However, they can easily pass into the brain and cause significant sedative effects, which limit their usefulness. Recently, our laboratory has synthesized zwitterionic derivatives of classical H_1-antagonists (1) with the aim of developing non-sedating H_1-antagonists as new antiallergic drugs. The sedative effect is caused by H_1-receptor blockade in the central nervous system (CNS). To properly evaluate the usefulness of the zwitterionic derivatives, we need to know the mechanisms by which H_1-antagonists are transported through the blood-brain barrier (BBB).

In a previous study (2), we suggested that H_1-antagonists penetrate into the brain *in vivo* via a carrier-mediated transport system. In order to characterize the transport mechanism under simpler, more controllable conditions, we have examined the transport mechanism of mepyramine as a model H_1-antagonist, using an *in vitro* system of primary cultured monolayers of bovine brain capillary endothelial cells (BCEC).

MATERIALS AND METHODS

Chemicals.

[^{3}H]Mepyramine (28.0 Ci/mmol) was purchased from Amersham (Buckinghamshire, England); L-[3, 4, 5-^{3}H(N)]leucine (153 Ci /mmol), 3-0-[methyl-^{3}H]-methyl-D-glucose (3-OMG) (79.0 Ci/mmol) and [^{14}C(U)]sucrose (5.0 mCi/mmol) from New England Nuclear (Boston, U.S.A.). All isotopes were stored at $-20°$ C until use. Horse serum was purchased from Hazleton Biologics Inc. (St. Lenexa, U.S.A.); dispase, collagenase/dispase, rat tail collagen type I and human fibronectin from Boehringer Mannheim GmbH (Mannheim, Germany); amphotericin B, gentamicin sulfate, Percoll, dextran (industrial grade MW. 87,000) and heparin from Sigma Chemical Co. (St. Louis, U.S.A.); polymixin B from Wako Pure Industries Ltd. (Osaka, Japan); mepyramine maleate from Sigma Chemical Co. (St. Louis, U.S.A.). All other chemicals were commercial products of reagent grade.

Isolation and culture of BCEC.

Capillary endothelial cells were isolated from bovine brains as reported (3), with minor modifications. Details of the preparation and culture were given in a previous report (4).

For cell culture, the frozen cells were thawed and washed 3 times with culture medium (pH 7.4, comprised of equal parts of minimum essential medium and F12-Ham's medium, containing 10 mM HEPES, 13 mM sodium bicarbonate, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, 100 μg/ml heparin, 50 μg/ml polymixin B and 10% horse serum), and seeded on collagen/fibronectin-coated culture dishes (4-well multidish, Nunc, Denmark) for the cellular uptake study. For determination of transendothelial permeability, prepared cells were seeded on collagen membrane-attached polystyrene chambers (Koken Cellgen, Koken, Tokyo, Japan). Cells were allowed to attach and grow to a monolayer at 37° C in a water-saturated atmosphere of 5% CO_2/95% air. After 3 days in culture, the culture medium (without polymixin B) was changed every other day. Uptake experiments were performed when the cells reached confluence, in approximately 10–12 days.

Cellular uptake experiments.

Uptake studies of ^{3}H- or ^{14}C-labeled compounds into cultured monolayers of BCEC were performed using reported methods (5) with minor modifications. Briefly, cultured cells were first washed 3 times with 1 ml of incubation solution (10 mM HEPES/NaOH buffer: 122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$,

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0.4 mM K_2HPO_4, 10 mM D-glucose, 10 mM HEPES, pH 7.4, 300 mOsm) at 37° C. In the case of 3-0-[^{3}H]MG uptake, D-glucose was omitted from the incubation solution. Uptake was initiated by adding 250 μl of incubation solution containing a ^{3}H- or ^{14}C-labeled compound (1.0 μCi) to cells in the absence or presence of various compounds. At designated times after the start of incubation, cells were washed 3 times with 1 ml of the ice-cold incubation solution, and uptake was terminated. To solubilize cells, 300 μl of 1 N NaOH was added to each dish and incubated at room temperature for 60 min. After neutralization with 300 μl of 1 N HCl, 500 μl of each solution was placed in a glass scintillation vial containing 10 ml of Clear-sol I. The radioactivity was then measured by a liquid scintillation counter, LSC-1,000 (Aloka Co. Ltd., Tokyo, Japan). Protein content of cultured cells per dish was determined by the Lowry method (6) using bovine serum albumin as a standard. Net uptake was expressed as the cell-to-medium concentration (cell/medium) ratio per mg protein, as follows:

$$\text{Cell/Medium Ratio } (\mu\text{l/mg protein}) = \frac{\text{Apparent uptake amount of substrate in BCEC}}{\text{Substrate concentration in the medium}} - \begin{array}{l}\text{Volume of medium absorbed}\\ \text{by BCEC measured using}\\ [^{14}\text{C}]\text{sucrose}\end{array}$$

In order to estimate the kinetic parameters of [^{3}H]mepyramine uptake in cultured monolayers of BCEC, the uptake rate (J) was fitted to the following equation, consisting of two saturable terms and a non-saturable linear term, using the nonlinear least-square regression analysis program, MULTI (7):

$$J = J_{max,1}\, s/(K_{t,1} + s) + J_{max,2}\, s/(K_{t,2} + s) + K_d\, s \qquad (1)$$

where J_{max} is the maximum uptake rate for a carrier-mediated process, s is the concentration of substrate, K_t is the half-saturation concentration (Michaelis constant), subscript integers indicate the saturable processes (1: high affinity, 2: low affinity), and K_d is the nonsaturable uptake rate constant. The transport data are presented as mean values ± S.E.

Effect of metabolic inhibitors on [^{3}H]mepyramine uptake.

Cultured cells were preincubated for 20 min at 37° C in the incubation solution in the absence or presence of metabolic inhibitors. The uptake of [^{3}H]mepyramine was measured at 37°C for 10 sec by incubating BCEC in 10 mM HEPES/NaOH buffer (pH 7.4) containing an inhibitor. The uptake of [^{3}H]mepyramine was determined by the method described above.

Transstimulation of [^{3}H]mepyramine uptake.

Cultured cells were preincubated for 20 min in the incubation solution containing 25 μM rotenone (to deplete cellular ATP) in the absence or presence of 250 μM unlabeled mepyramine. The uptake of [^{3}H]mepyramine was determined by the method described above. The significance of differences was assessed by using Student's *t* test.

Measurement of transcellular permeability coefficient of [^{3}H]mepyramine.

Koken Cellgen chambers containing cultured monolayers of BCEC were washed 3 times with 1 ml of incubation solution at 37° C, and placed in 24-well culture dishes maintained at 37° C. Measurement was initiated by adding 250 μl of incubation solution containing [^{3}H]mepyramine (2.0 μCi) and [^{14}C(U)] sucrose (0.4 μCi) to the donor chamber, and 500 μl of incubation solution to the receiving chamber. At designated times, 150 μl of solution was sampled from the receiving chamber and then 150 μl of incubation solution was added. The radioactivity of the sampled solution was measured by the method described above. Solubilization and the measurement of protein content of culture cells were also conducted as described above. Transcellular permeability coefficient was calculated as reported (8) with some modifications. The radioactivity that had permeated from the donor to the receiving chamber in each time interval was calculated by correcting for the radioactivity sampled from the receiving chamber at the preceding time points, and then converted into the equivalent volume cleared from the donor chamber at each time point. Four wells containing cultured monolayers of BCEC and 4 control wells were assayed for each condition. The average volume was plotted vs. time, and the slope (equal to the permeability coefficient, P_{tot}) was estimated by linear regression analysis.

The transcellular permeability coefficient (P_{trans}) was calculated as follows. The permeation process across monolayers of BCEC involves the diffusion in each chamber, the permeability through the monolayer of BCEC, which consists of transcellular and paracellular permeation processes, and the permeability of the filter. These processes can be expressed by the following equations.

$$\frac{1}{P_{tot}} = \frac{1}{P_{uns,d}} + \frac{1}{P_{trans} + P_{para}} + \frac{1}{P_{filt}} + \frac{1}{P_{uns,r}} = \frac{1}{P_{trans} + P_{para}} + \frac{1}{P_{filt,app}} \qquad (2)$$

The permeability coefficient of [^{3}H]mepyramine measured in monolayers of BCEC is denoted by P_{tot} and that measured in the fibronectin-coated collagen filter is denoted by $P_{filt,app}$. The subscript app indicates the apparent permeability coefficient including the diffusion coefficients of the unstirred water layer on the donor side ($P_{uns,d}$) and the receiving side ($P_{uns,r}$).

The permeability coefficient of [^{14}C]sucrose measured in monolayers of BCEC is denoted by $P_{tot,suc}$ and that measured in the fibronectin-coated collagen filter is denoted by $P_{filt,app,suc}$. The subscript app has the same meaning as above.

$$\frac{1}{P_{tot,suc}} = \frac{1}{P_{para}} + \frac{1}{P_{filt,app,suc}} \qquad (3)$$

From equations (2) and (3), the transcellular permeability coefficient can be calculated by means of equation (4).

$$P_{trans} = \frac{P_{tot} \cdot P_{filt,app}}{P_{filt,app} - P_{tot}} - \frac{P_{tot,suc} \cdot P_{filt,app,suc}}{P_{filt,app,suc} - P_{tot,suc}} \qquad (4)$$

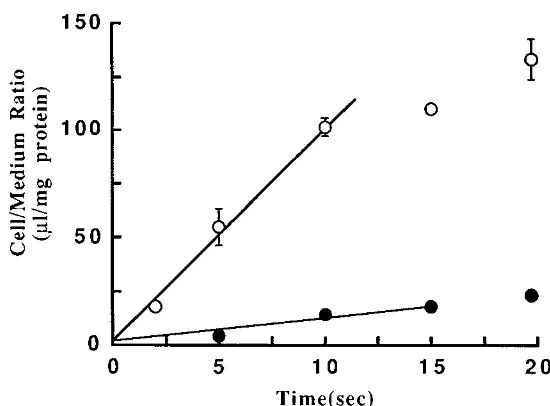

Fig. 1 Time course of [^{3}H]mepyramine uptake by cultured monolayers of BCEC at 37° C (open circles) and at 4° C (filled circles). Uptakes of [^{3}H]mepyramine were measured by incubating BCEC in 10 mM HEPES/NaOH buffer (pH 7.4). Each point represents the mean ± S.E. of three experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol.

The permeability coefficient is expressed as microliters per minute per mg protein (µl/min/mg protein).

RESULTS AND DISCUSSION

Primary cultured bovine brain endothelial cells are a useful model for studying the transport mechanism of nutrients (4,9) and drugs (4,10) because of their high viability and polarization of the membrane. We employed this *in vitro* model to investigate the transport mechanism of H_1-antagonists through the BBB. Mepyramine, the model compound used in this study, shows considerable transport and distribution into the brain (11), but the mechanisms involved remain unclear. We found a wide variation of permeability of H_1-antagonists depending on their chemical structures (unpublished observation). In particular, the permeability of carboxylated H_1-antagonists was low. Once the transport mechanism of H_1-antagonists is known, design of drugs that cannot be transported through the BBB should become possible, so the CNS side effects can be eliminated.

Permeation through the luminal membrane of the endothelium is the first step in crossing the BBB. A cellular uptake method is available to measure this process (4). In this culture system, the abluminal membrane of BCEC is allowed to attach to the culture dishes (12), making it easy to measure transport via the luminal membrane. The uptake coefficient of [^{3}H]mepyramine at the luminal membrane was 609 µl/mg protein/min (Fig. 1), which is similar to the calculated value of the transcellular permeability coefficient, 488 µl/mg protein/min (Table I). This similarity suggests that the rate-limiting step in the BBB transport of [^{3}H]mepyramine lies at the luminal membrane, so the characteristics of uptake at the luminal membrane may reflect well those of the transcellular transport process.

The time courses of [^{3}H]mepyramine uptake at 37° C and 4° C into the primary cultured monolayers of BCEC are shown in Fig. 1. The zero-time uptake of [^{3}H]mepyramine at 37° C and 4° C was close to the origin, suggesting that there was no significant binding to the membrane surface of BCEC, and that the uptake in the initial 10-sec period reflects the rate of uptake. Since metabolic inhibitors did not

Table I. Effect of unlabeled mepyramine on the transcellular permeability coefficient of [^{3}H]mepyramine.
Koken Cellgen system

Concn.	P_{filt}[a]	P_{tot}[a]	$P_{filt,suc}$[a]	$P_{tot,suc}$[a]	P_{para}[b]	P_{trans}[b]
Tracer	52.4	47.7	40.6	21.3	45.0	488
50 µM	53.2	48.7	41.6	21.9	46.4	520
125 µM	55.1	47.6	43.1	28.4	83.2	263
250 µM	55.3	47.5	42.5	31.2	118	220

Values are expressed in µl/min/mg protein.
[a]Each value represents the mean of three wells.
[b]Values were calculated from mean values of the permeability coefficient of three wells containing cell layers and three control wells for each condition.

Table II. Effect of metabolic inhibitors on the uptake of [^{3}H]mepyramine.

Inhibitor	Concn.	Relative Uptake[a]
	mM	%
Control		100.0 ± 4.4
DNP	0.25	93.6 ± 2.1
Rotenone	0.025	96.5 ± 5.2
KCN	1.0	102.2 ± 3.4
NaN_3	1.0	99.9 ± 5.5
Ouabain	0.5	104.4 ± 6.8

[a]Each value represents the mean ± S.E. of four experiments.

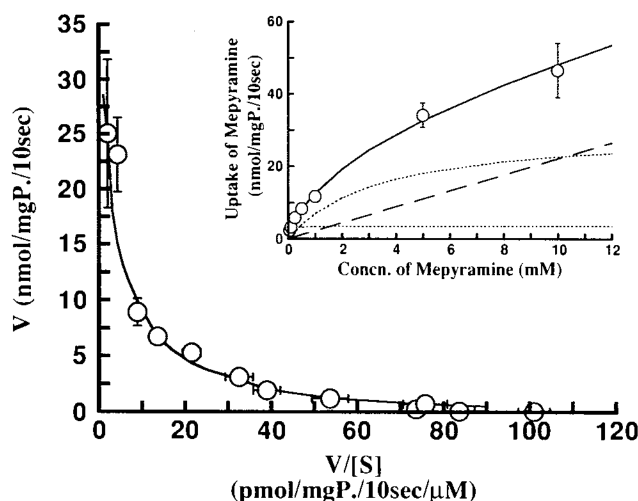

Fig. 2 Eadie-Hofstee plot of [^{3}H]mepyramine uptake by cultured monolayers of BCEC. Initial uptake rate of [^{3}H]mepyramine and unlabeled mepyramine (0.16 µM-10.0 mM) was measured at 37° C for 10 sec. Each point represents the mean ± S.E. of three experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol. Insert: Each point represents the mean ± S.E. of three experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol. The solid line represents total uptake rate generated from Eq. (1), using the MULTI fitted parameters (mean ± S.D.): $K_{t,1}$ = 49.8 ± 18.1 µM, $J_{max,1}$ = 3.52 ± 0.86 nmol/mg protein/10 sec, $K_{t,2}$ = 3.33 ± 0.95 mM, $J_{max,2}$ = 30.2 ± 0.85 nmol/mg protein/10 sec, K_d = 2.21 ± 0.17 µl/mg protein/10 sec. The dotted lines and dashed line represent saturable and nonsaturable uptake rates, respectively.

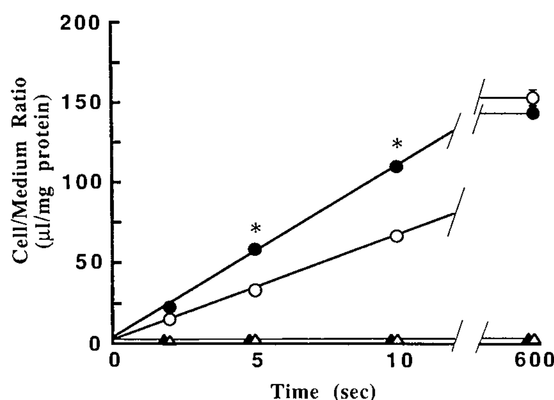

Fig. 3 Transstimulation effect on [^{3}H]mepyramine uptake by ATP-depleted cultured monolayers of BCEC. Cultured monolayers of BCEC had been preincubated for 20 min in the absence (open symbols) or presence (filled symbols) of mepyramine (250 µM) with rotenone (25 µM), and the uptakes of [^{3}H]mepyramine (circles) and [^{14}C]sucrose (triangles) was measured. Each point represents the mean ± S.E. of four experiments. When the S.E. is not indicated by a bar, it is smaller than the symbol. *Significantly different from the uptake of [^{3}H]mepyramine in the case without preincubation with mepyramine ($p < 0.001$).

affect the uptake of [^{3}H]mepyramine at 10 sec, the transport of [^{3}H]mepyramine requires no metabolic energy (Table II). Moreover, reduction of the incubation temperature from 37° C to 4° C decreased the initial uptake by 85%

Fig. 2 illustrates the relationship between the initial uptake and the concentration of mepyramine (0.16 µM to 10.0 mM). The result suggests that uptake of mepyramine consists of a saturable process and a nonsaturable process. The data were analyzed by use of the Eadie-Hofstee plot, as shown in Fig. 2. At least two different types of transport processes were apparent. The (J_{max}/K_t) value of the high-affinity process is 7.8 times greater than that of the low-affinity one, suggesting that the high-affinity process, $K_{t,1}$ = 49.8 µM, dominates at relatively low luminal concentrations of less than the $K_{t,1}$ value. To confirm that [^{3}H]mepyramine is transported across the plasma membrane via a carrier-mediated system at the luminal side of BCEC, transstimulation studies were carried out (13). The initial uptake of [^{3}H]mepyramine was significantly enhanced by preloading unlabeled mepyramine in the ATP-depleted cells. However, the uptakes of [^{14}C]sucrose (Fig. 3), 3-0-[^{3}H]MG and L-[^{3}H]leucine (data not shown) were not affected. These results again suggest that [^{3}H]mepyramine is transported via a specific carrier system into the BCEC.

A further experiment using the Koken Cellgen system showed that the transcellular permeability of [^{3}H]mepyramine was decreased concentration-dependently by addition of unlabeled mepyramine (Table I), supporting the involvement of a carrier-mediated transport system(s). However, the paracellular permeability measured using [^{14}C]sucrose was increased. This might be due to the weakness of intercellular junctions of BCEC.

In a previous study (2), we concluded on the basis of an *in vivo* brain uptake study that mepyramine permeates into the brain via a carrier-mediated transport system. The present results support the previous conclusion. The K_t values obtained *in vivo* (K_t = 4.40 mM) and *in vitro* ($K_{t,1}$ = 49.8 µM and $K_{t,2}$ = 3.33 mM) are different, but this may reflect the facts that we cannot find the $K_{t,1}$ value (high affinity and low capacity) in the *in vivo* brain uptake study because of its lower limit of detection.

We conclude that mepyramine, used here as a model of H_1-antagonists, is transported into the brain by a carrier-mediated transport system which does not require metabolic energy, probably via a facilitated diffusion mechanism, and the rate-limiting step is transport across the luminal membrane.

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