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Choline Uptake by Mouse Brain Capillary Endothelial Cells in Culture

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Abstract

Choline, a precursor of the neurotransmitter acetylcholine, is synthesized in only small amounts in the brain, so the choline concentration in the brain may vary depending on the plasma concentration and the transport rate across the blood–brain barrier. To elucidate the transport mechanism of choline, we carried out uptake experiments with mouse brain capillary endothelial cells in culture (MBEC4).

[^{3}H]Choline uptake was linear for up to 5 min. An examination of the concentration dependence of [^{3}H]choline uptake revealed the operation of both saturable ($J_{max} = 423 \pm 27$ pmol min^{-1} (mg protein)$^{-1}$ and $K_t = 20{\cdot}0 \pm 3{\cdot}1$ μM) and non-saturable ($k_d = 1{\cdot}23 \pm 0{\cdot}045$ μL min^{-1} (mg protein)$^{-1}$) processes. The saturable process was independent of Na^+ and pH, but was dependent on membrane potential as a driving force. Various basic drugs and endogenous substances, including substrates and inhibitors of the organic cation transporter, significantly inhibited the [^{3}H]choline uptake.

These data suggest that choline was taken up into the endothelial cells via two routes and that a membrane potential-dependent carrier-mediated transport system may participate in choline transport across the blood–brain barrier.

Brain capillary endothelial cells are tightly bound to each other and constitute the blood–brain barrier. To translocate drugs and endogenous substrates from the blood into the brain, various transporters exist on the endothelial cell membrane (Spector 1989; Terada & Tsuji 1991). The function of these selective transport systems helps to maintain the homoeostasis of the brain.

Choline is a precursor of the neurotransmitter acetylcholine, and is an essential constituent of cell membranes (Blusztajn & Wurtman 1983), but only small amounts are synthesized in the brain, and most of the brain's requirement is supplied from the diet (Wurtman 1992). It is therefore expected that the choline concentration in the brain should be affected by changes in the plasma choline concentration and the transport activity across the blood–brain barrier. It has been reported that choline is transported across the blood–brain barrier by facilitated diffusion (Cornford et al 1978), but little is known about choline uptake across the luminal membrane of endothelial cells (Estrada et al 1990).

Recently, an organic cation transporter (OCT1) has been cloned from rat kidney. OCT1 is located at the basolateral membrane of renal proximal tubules and transports various organic cations, including basic drugs such as tetraethylammonium (TEA) and *N*-methylnicotinamide, from the blood into the renal epithelial cells. Its transport activity depends on the membrane potential and is independent of Na^+ or pH (Hori et al 1992; Grundemann et al 1994; Nelson et al 1995). It has been reported that choline is transported by OCT1 in the kidney (Busch et al 1996b). Therefore OCT1 or a related molecule may also transport choline at the blood-brain barrier. The relationship between various basic drugs and the putative choline transporter has been examined by using a rat brain uptake index method or bovine isolated brain capillary (Kang et al 1990).

In this study, mouse brain capillary endothelial cells in culture (MBEC4) were used to investigate the functional properties of choline uptake on the luminal membrane of endothelial cells. The effects

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of various basic drugs on choline uptake were studied also.

Materials and Methods

Chemicals

[^{3}H]Choline chloride (sp. act. 83·0 Ci mmol^{-1}) was purchased from Amersham International plc (Buckinghamshire, UK). [^{14}C]Sucrose was purchased from du Pont New England Nuclear (Boston, MA). Choline chloride, hemicholinium-3 and *N*-methylnicotinamide were from Aldrich Chemical Company Inc. (Milwaukee, WI). Cimetidine and procainamide hydrochloride were from Sigma Chemical Co. (St Louis, MO). Quinine sulphate, TEA, guanidine hydrochloride, L-arginine, L-lysine monohydrochloride, *p*-aminohippuric acid, acetylcholine, dopamine chloride, 5-hydroxytryptamine (5-HT) hydrochloride and histamine dihydrochloride were from Nacalai Tesque Inc. (Kyoto, Japan). Bunitrolol was kindly supplied by Boehringer Ingelheim (Ingelheim am Rhein, Germany). All other chemicals were commercial products of reagent grade.

Cell culture

Mouse brain capillary endothelial cells (MBEC4) were established in our laboratory as described by Tatsuta et al (1992). MBEC4 cells were routinely grown in 75-cm^2 plastic T-flasks at 37°C in a 5% CO_2/95% air atmosphere. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Nikken Bio Medical Lab., Kyoto, Japan) supplemented with 10% foetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. For the uptake study, the MBEC4 cells were seeded at a density of 4×10^4 cells mL^{-1} on 4-well multi dishes (Nunc, Denmark).

Uptake measurements

MBEC4 cells grown on the 4-well multi dishes were rinsed three times with 1 mL incubation buffer containing (mM): 141 NaCl, 4 KCl, 2·8 $CaCl_2$, 1 $MgSO_4$, 10 D-glucose, 10 HEPES (pH 7·4). The uptake study was initiated by adding 250 μL incubation buffer containing [^{3}H]choline (5 nM). The cells were incubated at 37°C for the time designated. To terminate uptake, the cells were washed three times with ice-cold incubation buffer. For the quantitation of the radioactivity associated with the cells, the cells were solubilized with 250 μL 1 M NaOH. After neutralization with 250 μL 1 M HCl, the sample was placed in a scintillation vial, and 4 mL scintillation fluid was added. The concentration of [^{3}H]choline was measured with an LS6500 (Beckman Instruments Inc., CA). The extracellular space was determined in terms of [^{14}C]sucrose uptake. Cellular protein content was measured by the method of Lowry et al (1951) using bovine serum albumin as the standard. Details of the conditions of each experiment are presented in the legends of the figures or table footnotes.

Data analysis

To estimate the kinetic parameters of [^{3}H]choline uptake in MBEC4 cells, the uptake rate (J) was fitted to equation 1, which has both saturable and non-saturable linear terms, using the non-linear least-squares regression analysis program, MULTI (Yamaoka et al 1981):

$$J = \frac{J_{max} \times S}{K_t + S} + K_d \times S \qquad (1)$$

where J_{max} is the maximum uptake rate for the saturable component, K_t is the Michaelis constant, k_d is the first-order constant for the non-saturable component and S is the concentration of choline.

Data are expressed as mean ± s.e. A statistical analysis was performed by using Student's two-tailed *t*-test. The difference between means was considered to be significant when $P < 0.05$.

Results

Characteristics of [^{3}H]choline uptake by MBEC4 cells

Choline uptake by MBEC4 cells was linear for up to 5 min (data not shown). The incubation time was set as 1 min in all experiments because choline may be metabolized in the cytosol (Estrada et al 1990). Within this incubation time, most [^{3}H]choline remains intact in the cells (Estrada et al 1990). The cell-to-medium ratio of [^{3}H]choline uptake was 20·8 ± 0·2 μL (mg protein)$^{-1}$ at 1 min, while the cell volume of MBEC4 cells was approximately 3 μL (mg protein)$^{-1}$ (our data). Thus, MBEC4 concentrated [^{3}H]choline by a factor of seven over the extracellular level.

Figure 1 shows the relationship between the initial uptake rate and the concentration of choline (5 nM – 1 mM). Analysis of these data using equation 1 suggested the operation of two transport processes, one saturable carrier-mediated system and the other non-saturable, within the concentration

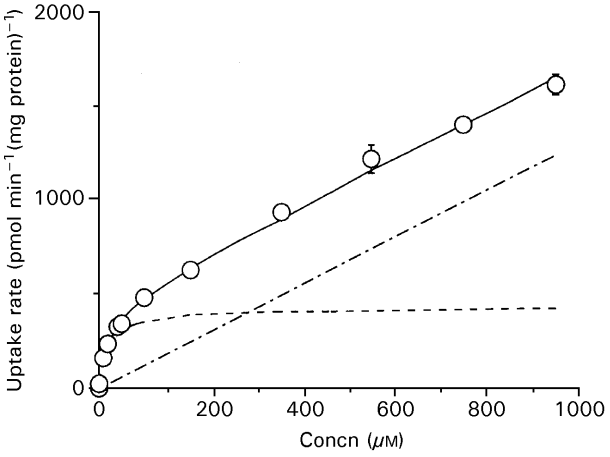

Figure 1. Concentration dependence of choline uptake by MBEC4 cells. Initial uptake rates at various concentrations of choline (5 nM–1 mM) were measured at 37°C for 1 min. Each point represents the mean ± s.e. for four experiments. Curves for total uptake (——), saturable component (- - -) and non-saturable component (– – –) were drawn by using the parameters obtained from non-linear regression analysis (MULTI).

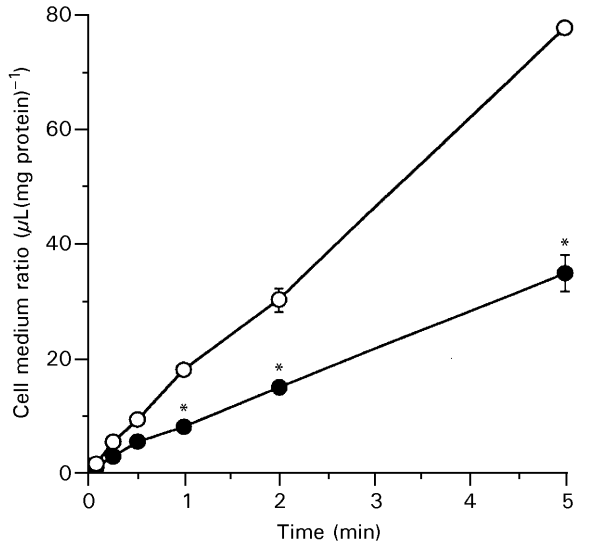

Figure 2. Effect of membrane potential on [^{3}H]choline (5 nM) uptake by MBEC4 cells. [^{3}H]Choline uptake was measured at 37°C in incubation buffer (○) or buffer in which Na^+ was replaced by K^+ and which contained 10 μM valinomycin (●). Each point represents the mean ± s.e. for three to four experiments. *$P < 0.05$ compared with the control.

range used. Using equation 1 the following kinetic parameters were obtained: $J_{max} = 423 \pm 27$ pmol min^{-1} (mg protein)$^{-1}$, $K_t = 20{\cdot}0 \pm 3{\cdot}1$ μM, and $k_d = 1{\cdot}23 \pm 0{\cdot}045$ μL min^{-1} (mg protein)$^{-1}$. These parameters suggest that approximately 94·5% of the choline was taken up via the saturable system at 5 nM [^{3}H]choline (Figure 1).

When MBEC4 cells were depolarized by replacement of Na^+ with K^+ in the incubation buffer and addition of 10 μM valinomycin, an ionophore of K^+, the uptake of [^{3}H]choline was significantly

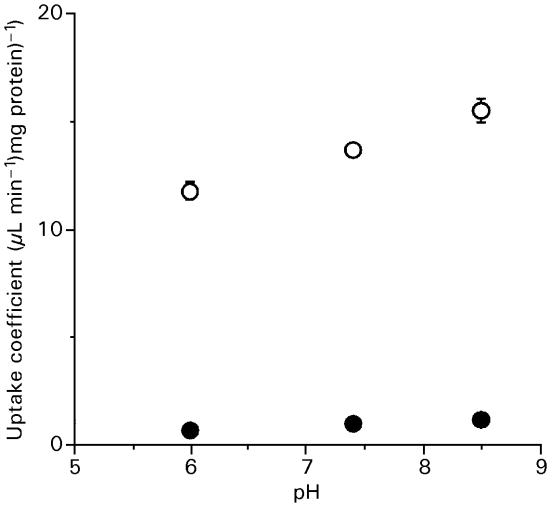

Figure 3. Effect of pH on [^{3}H]choline (5 nM) uptake by MBEC4 cells. [^{3}H]Choline uptake was measured at 37°C for 1 min at various pH in the absence (○) and presence of 500 μM unlabelled choline (●). Each point represents the mean ± s.e. for four experiments.

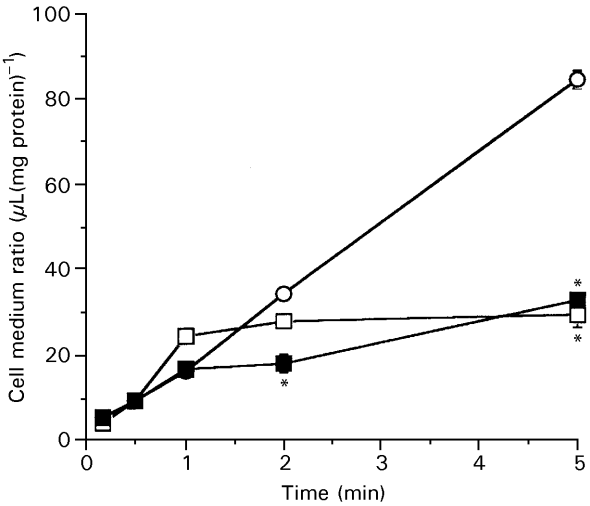

Figure 4. Effect of metabolic inhibitors on [^{3}H]choline (5 nM) uptake by MBEC4 cells. After MBEC4 cells were pre-incubated for 10 min with (□, 10 mM NaN_3; ■, 50 μM FCCP) or without (○) a metabolic inhibitor, and the [^{3}H]-choline uptake was measured at 37°C with or without metabolic inhibitor. Each point represents the mean ± s.e. for three to four experiments. *$P < 0.05$ compared with the control.

decreased (Figure 2). This finding indicated the membrane potential dependence of [^{3}H]choline uptake. The [^{3}H]choline uptake was not affected by replacement of external sodium with lithium or *N*-methylglucamine or by change of the pH (Table 1, Figure 3). In the presence of 0·1 mM ouabain, a Na^+-K^+ ATPase inhibitor, [^{3}H]choline uptake was unchanged (Table 1). The effect of metabolic inhibitors on [^{3}H]choline uptake is shown in Figure

Table 1. Effect of Na^+ and ouabain on [^{3}H]choline uptake by MBEC4 cells.

Treatment	Cell-to-medium ratio (% of control)
Control	100 ± 12·7
Na^+ replaced by Li^+	117 ± 0·45
Na^+ replaced by *N*-methylglucamine$^+$	87·4 ± 2·66
+ 0·1 mM Ouabain	123 ± 2·04

For investigation of sodium dependency, [^{3}H]choline uptake was measured at 37°C for 1 min where Na^+ in the incubation buffer was replaced by Li^+ or *N*-methylglucamine$^+$. For ouabain, MBEC4 cells were pre-incubated for 10 min with ouabain, and the [^{3}H]choline uptake was measured at 37°C with ouabain for 1 min. Each value represents the mean ± s.e. for three to four experiments.

4. In the presence of 10 mM sodium azide or 50 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP), [^{3}H]choline uptake showed no change within 1 min, but was significantly decreased at 5 min.

Effects of various drugs on [^{3}H]choline uptake

The effects of various basic drugs, basic amino acids, an organic anion and neurotransmitters on [^{3}H]choline uptake were examined. As shown in Figure 5, basic drugs significantly inhibited [^{3}H]choline uptake, and the order of inhibitory potency was quinine > guanidine > hemicholinium-3 > bunitrolol > TEA > *N*-methylnicotinamide > cimetidine > procainamide. Two basic amino acids (L-lysine and L-arginine) and an organic anion (*p*-aminohippuric acid) had no effect on the [^{3}H]choline uptake (data not shown). Neurotransmitters reduced [^{3}H]choline uptake, and the inhibitory potency was in the order of acetylcholine > 5-HT > dopamine > histamine. The inhibitory potency of acetylcholine, a choline analogue, was extremely high (Figure 5).

Trans-stimulation of [^{3}H]choline uptake and counter-transport effects

After pre-incubation of MBEC4 cells with unlabelled choline or a basic drug such as cimetidine, quinine, procainamide or TEA for 10 min under a condition of ATP depletion, trans-stimulation of [^{3}H]choline uptake and counter-transport effects were examined. As shown in Table 2, the [^{3}H]choline uptake was significantly stimulated in the presence of unlabelled choline.

Discussion

The mechanism of choline uptake across the luminal membrane of MBEC4 cells was investigated. [^{3}H]Choline uptake appeared to involve both saturable and non-saturable processes (Figure 1). The plasma choline concentration in healthy adults is approximately 10 μM (Cohen et al 1995). If the kinetic parameters of choline in MBEC4 cells are the same as those in the human brain, approximately 90% of the choline incorporated into the human brain enters via carrier-mediated transport, at this concentration.

We demonstrated that choline was concentratively accumulated in brain capillary endothelial cells. However, the brain choline level is approximately 2·5 μM, one-quarter of that in plasma (Tucek 1984). In cerebral capillary endothelial cells with intact endocellular choline pools, [^{3}H]choline was only partially metabolized and 63% of accumulated tritium remained in free choline (Estrada et al 1990). Also, it was confirmed that only a small fraction of the intracellular choline was transported back into the external medium from cerebral capillary endothelial cells (Estrada et al 1990). These results suggest that brain capillary endothelial cells may have a mechanism to limit the supply of choline into the brain.

Cornford et al (1978) examined choline uptake across the blood–brain barrier in rats. The value of K_t was 310 μM, fifty times our present value (20 μM). However, those authors measured net transport of choline across both luminal and abluminal sides of endothelial cells in rats, so their result is not comparable with ours.

Based on the Na^+-independency and the hemicholinium-3 sensitivity observed in this study, the choline transporter in MBEC4 cells appears to be different from the two choline transporters on the cholinergic neurons, high-affinity choline uptake, which is a Na^+-dependent transporter, and low-affinity choline uptake, which is insensitive to hemicholinium-3 (Yamamura & Snyder 1973; Kuhar & Murrin 1978; Jope 1979). Choline itself is also a substrate of OCT1 in the kidney (Busch et al 1996b). Our data show that the brain choline uptake transporter resembles the OCT family (OCT1, OCT2) in some functional properties, as well as its dependence on membrane potential and independence of Na^+ and pH. These findings suggest a close similarity between the choline transporter and OCTs. Most of the basic drugs used in this study are known to be substrates or inhibitors of OCTs, they may also be transported by the choline transporter in the blood–brain barrier. OCT1 is located in the kidney, liver and small intestine, but not the brain (Grundemann et al 1994), while OCT2 is located only in the kidney (Okuda et al 1996). Therefore the choline transporter on MBEC4 cells seems to be distinct from OCT1 or OCT2, but might be a different subtype of the OCT family.

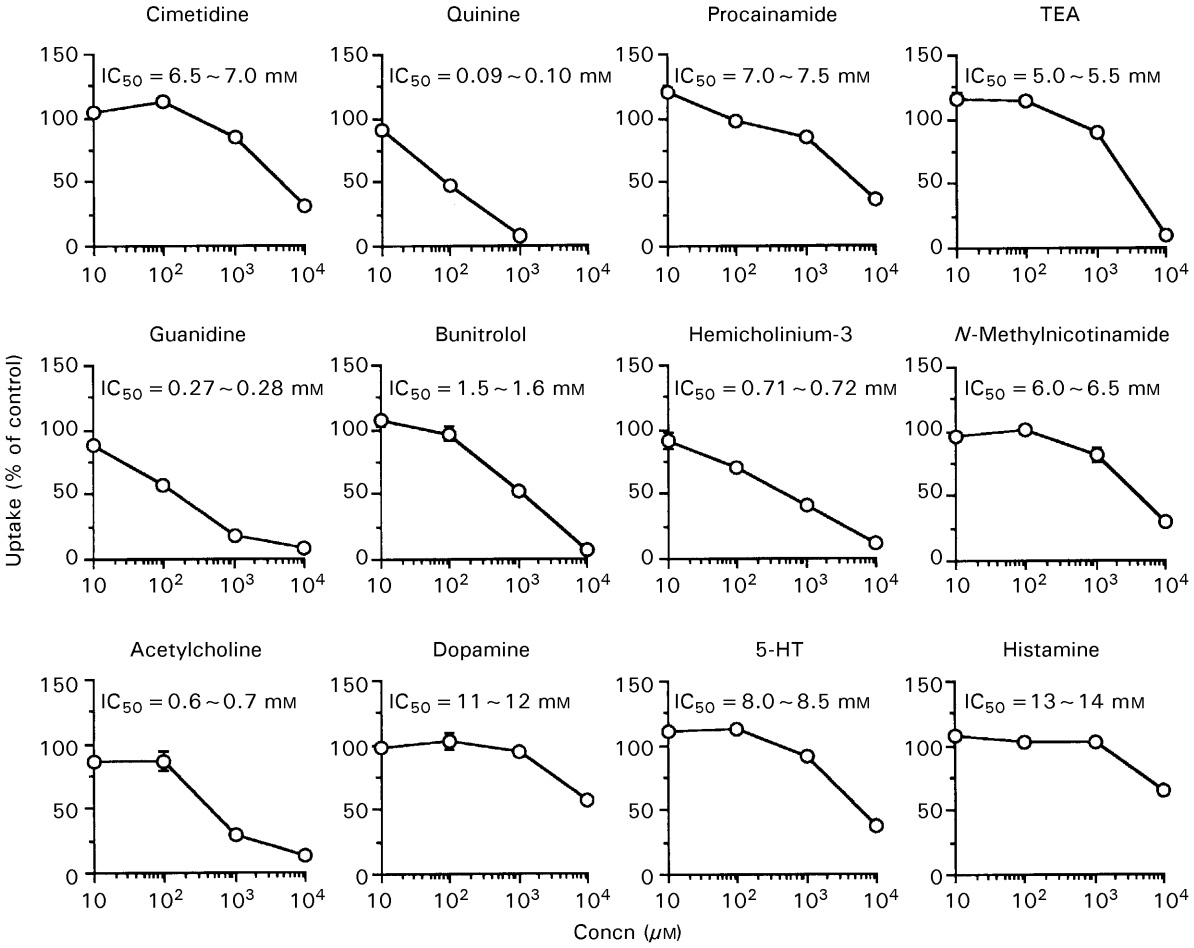

Figure 5. Effect of various drugs on [^{3}H]choline (5 nM) uptake by MBEC4 cells. [^{3}H]Choline uptake (% of control) was measured at 37°C for 1 min in the absence and presence of various drugs. Each point represents the mean ± s.e. for four experiments. Apparent IC50 (half-inhibitory concentration) was estimated from each inhibition curve.

Neurotransmitters are transported by OCT1 in the kidney (Busch et al 1996a). Accordingly, our choline transporter on the brain capillary endothelial cells may transport some neurotransmitters. Several neurotransmitters indeed decreased the choline uptake, and the inhibitory effect of acetylcholine was remarkably large (Figure 5). Nevertheless acetylcholine was not taken up by MBEC4 cells (data not shown). This finding suggests that acetylcholine simply binds to the choline transporter or that acetylcholine taken up by MBEC4 cells, is ejected via a separate mechanism.

[^{3}H]Choline uptake was decreased by metabolic inhibitors (Figure 4). When the cells are incubated with metabolic inhibitors, it is likely that the intracellular ion constitution cannot be maintained, and the consequent depolarization might be responsible for the reduction of [^{3}H]choline uptake.

[^{3}H]Choline uptake was also significantly decreased in the presence of basic drugs (Figure 5),

Table 2. Trans-stimulation of [^{3}H]choline (5 nM) uptake by ATP-depleted MBEC4 cells and counter-transport effects.

Drugs	Concn	Cell-to-medium ratio (% of control)
Control		100 ± 6·43
Choline	20 μM	139 ± 11·3*
	10 μM	128 ± 14·6
Cimetidine	10 mM	61·4 ± 8·36*
	5 mM	160 ± 25·9
Quinine	500 μM	24·8 ± 3·84*
	100 μM	44·8 ± 2·05*
Procainamide	10 mM	97·9 ± 28·4
	5 mM	90·7 ± 3·18
TEA	10 mM	87·4 ± 6·26
	5 mM	116 ± 5·65

MBEC4 cells were pre-incubated in ATP-depleted buffer (plus 10 mM sodium azide and minus glucose) alone or ATP-depleted buffer containing a drug at the indicated concentration, and the [^{3}H]choline uptake rate was measured at 37°C for 1 min in ATP-depleted buffer. Each value represents the mean ± s.e. for three to four experiments. *$P < 0·05$ compared with the control.

suggesting that the transporter participates in the uptake of these drugs. Winkle (1993) reported that choline was transported by a basic amino acid transporter. However, in our experiments, the basic amino acids L-arginine and L-lysine had no effect on [^{3}H]choline uptake by MBEC4 cells.

It has been reported that ouabain, an inhibitor of Na^+-K^+ ATPase, reduced the choline uptake by bovine brain isolated capillary (Galea & Estrada 1992), but in our study [^{3}H]choline uptake was not affected by ouabain. Because the previous workers used a cell suspension for the uptake study, the total choline uptake may include the uptake through the abluminal membrane.

Large amounts of unlabelled choline transstimulated the [^{3}H]choline uptake (Table 2), supporting the existence of a choline transporter. Cimetidine and TEA exhibited similar, though less marked, effects, suggesting that they may be transported by the same transporter. However, a high concentration of cimetidine or TEA (10 mM) reduced [^{3}H]choline uptake. Preloaded quinine reduced the choline uptake. Quinine showed a high inhibitory potency (Figure 5), and may bind strongly to the choline transporter.

In conclusion, our results suggest that choline is predominantly translocated across the blood-brain barrier via a carrier-mediated transport system, which is dependent upon membrane potential, but independent of Na^+ and pH. It remains to be determined whether the choline transport system is a member of the OCT family, and what role it plays in the transport of basic drugs across the blood-brain barrier.

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