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Transport of Ipratropium, an Anti-Chronic Obstructive Pulmonary Disease Drug, Is Mediated by Organic Cation/Carnitine Transporters in Human Bronchial Epithelial Cells: Implications for Carrier-Mediated Pulmonary Absorption

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Abstract: Ipratropium bromide, an anticholinergic drug used for the treatment of asthma and chronic obstructive pulmonary disease, has low oral bioavailability, but systemic exposure, superior to oral administration, can be achieved by inhalation. Therefore, we investigated the pulmonary absorption mechanism of ipratropium using human bronchial epithelial BEAS-2B cells. [^{3}H]Ipratropium uptake by BEAS-2B cells was temperature-dependent and saturable, with a K_m value of 78.0 μM, suggesting involvement of carrier-mediated uptake. An RT-PCR study showed that organic cation/carnitine transporters OCTN1 and OCTN2 are expressed in BEAS-2B cells, but organic cation transporters (OCTs) are not. Uptake of [^{3}H]ipratropium by HEK293 cells expressing OCTN1 (HEK293/OCTN1) and OCTN2 (HEK293/OCTN2) was significantly increased, compared with mock-transfected cells, and the estimated K_m values were 444 μM and 53.0 μM, respectively. Finally, the contributions of OCTN1 and OCTN2 to ipratropium uptake were evaluated by measuring [^{3}H]ipratropium uptake by BEAS-2B cells in which OCTN1 or OCTN2 gene expression had been silenced. Knock-down of OCTN1 or OCTN2 suppressed the uptake of [^{3}H]ipratropium to 78.2% and 14.8% of that by control BEAS-2B cells, respectively. In addition, another anticholinergic, tiotropium, was also taken up by both HEK293/OCTN1 and HEK293/OCTN2 cells. Therefore, ipratropium and tiotropium are taken up primarily by OCTN2, and to a lesser extent by OCTN1, in bronchial epithelial cells. These findings are consistent with the pharmacological activity of the drugs after administration via inhalation.

Keywords: Ipratropium; tiotropium; OCTN1; OCTN2; transporter; pulmonary absorption; carnitine

Introduction

Organic cation/carnitine transporters, OCTN1 (SLC22A4) and OCTN2 (SLC22A5), are physiologically important plasma membrane transporters involved in the distribution of organic cations and nutrients, such as carnitine[1−3] and ergothioneine.[4,5] We have shown that OCTN2 is essential for the β-oxidation of fatty acids, and loss of OCTN2 function leads to primary systemic carnitine deficiency.[2,3,6] OCTNs also play a role in the renal handling of pharmacologically important drugs.[7,8] Since OCTNs are highly expressed at the apical side of airway epithelial cells in tracheal tissue,[1,2,9] they might also play a role in the delivery

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of cationic drugs used for treatment of pulmonary diseases, such as asthma and chronic obstructive pulmonary disease (COPD).[9]

COPD is a major health problem and is the fourth leading cause of chronic morbidity and mortality in the United States.[10] Anticholinergic bronchodilators have been the agents of choice to treat patients with COPD for the last two decades, often combined with corticosteroids. Inhaled anticholinergics include tiotropium and ipratropium bromide, both of which act on muscarinic receptors.[11,12] It has been thought that inhaled anticholinergics are absorbed into the airway tissue by passive diffusion, since inhalation allows the tissue to be exposed to these anticholinergics at high concentrations.[13] However, superior systemic exposure to hydrophilic tiotropium and ipratropium can be achieved after a single dose delivered by inhalation,[14,15] and it has been postulated that active transport is involved.[16] In addition, ipratropium was shown to inhibit not only carnitine uptake by a human proximal tubular cell line, Caki-1, in which OCTN2 is abundantly expressed at the apical membranes,[17] but also MPP^+ uptake by OCT2-expressing HEK293 cells,[18] suggesting that *in vivo* distribution of ipratropium could be influenced by organic cation transporters, OCTs and OCTNs.

In the present study we evaluated ipratropium uptake by airway epithelial cells to establish whether active transport is involved in pulmonary absorption of ipratropium. Although relatively hydrophilic compounds more likely paracellularly cross the epithelium through aqueous pores in the intercellular tight junctions, carrier-mediated transport is known to be involved in entry of a number of compounds (e.g., disodium cromoglycate and cycloleucine) for systemic entry from airway epithelium.[13] Here, we present evidence that human bronchial epithelial BEAS-2B cells possess transporters to import ipratropium, among which OCTN2 plays a major role. Since the airway epithelium represents a barrier through which inhaled bronchodilators must pass to reach targeted receptors in the underlying airway smooth muscle, a better understanding of the pulmonary absorption mechanisms for ipratropium should provide information which can be used to develop more effective inhaled drugs for the treatment of COPD and asthma.

Experimental Section

Chemicals. Ipratropium and tiotropium bromide, and [^{3}H-methyl]ipratropium and [^{3}H-methyl]tiotropium bromide were provided by GlaxoSmithKline (Ware, UK). [^{3}H]Ergothioneine (3.7 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA), and L-[^{3}H]carnitine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Corticosterone and unlabeled ergothioneine were purchased from Sigma-Aldrich (St. Louis, MO). Tetraethylammonium chloride, quinidine sulfate, and unlabeled L-

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carnitine were obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

Cell Culture. HEK293 and BEAS-2B cells were obtained from American Type Culture Collection (Manassas, VA) and from DS Pharma Biomedical Co., Ltd. (Osaka, Japan), respectively. Both cell lines were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 units/mL penicillin, 100 μg/mL streptomycin, and at 37 °C in an atmosphere of 5% CO_2.

Transport Experiments with HEK293 and BEAS-2B Cells. OCTN1- and OCTN2-expressing HEK293 cells (designated as HEK293/OCTN1 and HEK293/OCTN2) were prepared as previously described.[19,20] HEK293 and BEAS-2B cells (5.0×10^4 cells/well) were seeded into 24-well plates and then cultured for 2 days. In general, uptake was initiated by adding 0.25 mL of transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, adjusted to pH 7.4) containing radiolabeled test substrate to cells of interest, and cells were incubated for 3 min, 5 min, and 30 s to evaluate ipratropium uptake by BEAS-2B, OCTN1 and OCTN2, respectively, unless the period of the time is indicated. At the end of the uptake reaction, cells were washed with ice-cold transport buffer twice, and then solubilized in 0.2 mL of 1% (v/v) Triton X-100. The radioactivity in the resultant cell lysate was measured using a liquid scintillation counter (Aloka Co. Ltd., Tokyo, Japan). Part of the lysate was used for determination of total protein amount with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) as previously described.[21]

RNA Silencing Using Artificial Short-Hairpin RNA (shRNA). Knock-down of OCTN1 and OCTN2 was performed using the BLOCK-iT Pol II miR RNAi expression vector kit according to the protocol of the manufacturer (Invitrogen). Oligonucleotides targeted to OCTN1 and OCTN2 were purchased from Invitrogen, and subcloned into pcDNA 6.2-GW/EmGFP-miR, respectively, leading to bicistronic expression of Emerald GFP (EmGFP). The pcDNA6.2-GW/EmGFP-miR-negative control plasmid contains an oligonucleotide not related to OCTNs, which serves as a negative control. BEAS-2B cells were transfected with these plasmid DNAs using LipofectAMINE 2000 reagent (Invitrogen). Two days later, selection of transfected cells was started with blasticidin (10 mg/mL). After 3 weeks, blasticidin-resistant and emGFP-positive cells were used for the transport studies and subjected to RT-PCR analysis.

Table 1. Gene-Specific Oligonucleotide Primer Pairs

application	gene	primer sequence (5′ to 3′)
qRT-PCR	OCTN1	
	sense	CTGCTATTGCGAACCCTGCC
	antisense	CAGCATGACCAGACCAATGGATAAG
	OCTN2	
	sense	CAGCCATCCTCACCTTGTTTC
	antisense	TGTGGGCCTTTCTTGACCATC
	β-actin	
	sense	GCTATCCAGGCTGTGCTATC
	antisense	TGTCACGCACGATTTCC
RT-PCR	OCT1	
	sense	GATTTAAAGATGCTTTCCCTCGAA
	antisense	TCCCTCAGCCTGAAGACTATGAA
	OCT2	
	sense	GAAGCCATGAGAATCATTAAGCAC
	antisense	CCATTCTTCCCAAGCATGAGATAA
	OCT3	
	sense	TGATCATCTTTGGTATCCTGGCATC
	antisense	AACTTTCTCAAATCCTTGGTCGGCA
	OCTN1	
	sense	ACCTCAGTGGGTTACTTTGCTC
	antisense	GGTAGAGCTCAGCAGTGAAGAC
	OCTN2	
	sense	GGGCAAGTTTGGAGTCACGG
	antisense	AGCAAGTCAGACACAGGTCAAGAG

Reverse Transcription Polymerase Chain Reaction. Total RNA was prepared from BEAS-2B cells using ISOGEN according to the manufacturer's protocol (Nippon Gene, Toyama, Japan). Single-strand cDNAs were synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and the expression levels of OCT1, OCT2, OCT3, OCTN1 and OCTN2 mRNAs were analyzed by RT-PCR. Amplification conditions for PCR consisted of denaturation at 95 °C for 30 s, annealing at 56 or 60 °C for 30 s, and extension at 72 °C for 45 s for 35 cycles for OCT1, OCT2 and OCT3, or 30 cycles for OCTN1 and OCTN2. The PCR products were separated by electrophoresis in 2% agarose gel and visualized using Light Capture (Atto Co., Tokyo, Japan). Relative quantification of OCTN1 and OCTN2 mRNA expression was performed with an Mx3000p Real-Time PCR system (Stratagene, Cedar Creek, TX) using FullVelocity SYBR Green QPCR (Stratagene) with 50 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. The expression level was normalized to that of β-actin. Gene-specific primers used for RT-PCR are listed in Table 1.

Analytical Methods. Cell-to-medium ratio (μL/mg protein) was obtained by dividing the intracellular accumulation of test compound by its concentration in the transport medium. The apparent kinetic parameters, K_m (Michaelis constant), V_{max} (maximal uptake rate) and k_d (apparent nonsaturable first-order rate constant) of ipratropium uptake by BEAS-2B and HEK293 cells, were calculated by non-

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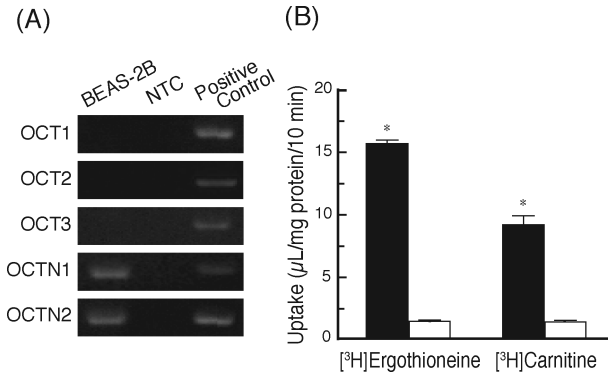

Figure 1. (A) Expression of several organic cation transporter mRNAs and (B) transport function of [^{3}H]-ergothioneine and [^{3}H]carnitine in BEAS-2B cells. (A) The reactions using specific primer sets were conducted as described in the Experimental Section. NTC stands for no template control (negative control). As positive controls, we used cDNAs derived from HepG2 cells for OCT1, ACHN cells for both OCT2 and OCTN2, CoLoTC cells for OCT3, and K562 cells for OCTN1. The PCR reaction was applied for the amplification of each gene as described in the Experimental Section. (B) Uptake of [^{3}H]ergothioneine (2 μM) and of [^{3}H]carnitine (0.1 μM) by BEAS-2B cells was measured for 10 min at 37 °C (filled bars) or 4 °C (unfilled bars). Data are shown as mean ± SEM (n = 3), and (*) indicates a significant difference from the uptake at 4 °C ($p < 0.05$).

linear least-squares regression analysis using KaleidaGraph 4.0.2 (Synergy Software, Reading, PA), according to the following Michaelis−Menten type equations, eqs 1 and 2, with and without a nonsaturable component, respectively, where v and s are the uptake rate of substrate and substrate concentration, respectively.

$$v = V_{max}s/(K_m + s) + k_d s \quad (1)$$

$$v = V_{max}s/(K_m + s) \quad (2)$$

All data were expressed as the mean ± SEM, and statistical analysis was performed by applying Student's t test. The criterion of significance was taken to be $p < 0.05$.

Results

Expression of Organic Cation Transporters in BEAS-2B Cells. We initially examined expression of organic cation transporters in BEAS-2B cells. As shown in Figure 1A, mRNA expression of OCT1, OCT2, OCT3, OCTN1, and OCTN2 was studied by means of RT-PCR. OCTN1 and OCTN2 mRNAs were detected in BEAS-2B cells, whereas OCT1, OCT2 and OCT3 mRNAs were not (up to 35 PCR cycles). Next, functional expression of OCTN1 and OCTN2 in BEAS-2B cells was investigated by incubating the cells with ergothioneine and carnitine for 10 min at 4 °C and at 37 °C. The results are shown in Figure 1B. Uptake of [^{3}H]ergothioneine, a specific substrate of OCTN1, and of [^{3}H]carnitine, a specific substrate of OCTN2, was increased

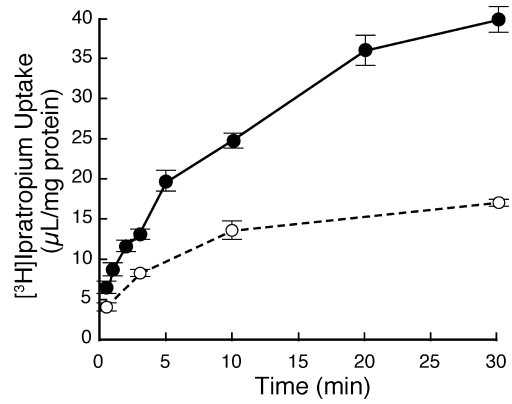

Figure 2. Time course of [^{3}H]ipratropium uptake by BEAS-2B cells. Uptake of [^{3}H]ipratropium (15 nM) by BEAS-2B cells was measured over 30 min at pH 7.4 and 37 °C (close circles) or 4 °C (open circles). Data are shown as mean ± SEM (n = 4).

9.1- and 6.5-fold, respectively, at 37 °C, compared with the that at 4 °C, suggesting that BEAS-2B cells express functionally active OCTN1 and OCTN2.

Transporter-Mediated Ipratropium Uptake by BEAS-2B Cells. To determine whether ipratropium is actively transported into airway epithelium, [^{3}H]ipratropium uptake was initially examined in BEAS-2B cells (Figure 2). Uptake of [^{3}H]ipratropium by BEAS-2B cells increased time-dependently for up to 5 min and reached a plateau within 30 min. [^{3}H]Ipratropium uptake at 4 °C was evident, but significantly lower than that at 37 °C, suggesting transporter-mediated uptake. To evaluate the uptake rate, we decided to quantify intracellular accumulation of [^{3}H]ipratropium for 3 min, which was in the linear range of uptake. Ipratropium uptake by BEAS-2B cells was saturable with a K_m of 78.0 ± 1.7 μM and V_{max} of 117 ± 1.4 pmol/mg protein/3 min according to eq 1 (Experimental Section). The first-order rate constant (k_d) for the apparently nonsaturable component was 0.87 ± 0.0014 μL/mg protein/3 min (Figure 3). In addition, organic cations, such as TEA and quinidine, and carnitine significantly reduced [^{3}H]ipratropium uptake by 26.8%, 66.5% and 21.7%, respectively, at the concentration of 1 mM (Table 2). These results suggest that an organic cation transporter mediates ipratropium uptake by BEAS-2B cells.

Ipratropium and Tiotropium Uptake by OCTN1- and OCTN2-Expressing HEK293 Cells. Based on the results of characterization of the expression of organic cation transporters in and ipratropium uptake by BEAS-2B cells, we hypothesized that ipratropium is transported into the cells via OCTN1 and/or OCTN2. To test this hypothesis, we compared [^{3}H]ipratropium uptake by HEK293/OCTN1 and HEK293/OCTN2 cells with that by HEK293 cells transfected with pcDNA3.1 alone (designated as MOCK) (Figure 4A,B). The uptake by HEK293/OCTN1 and HEK293/OCTN2 cells was significantly higher than that by MOCK cells, and increased time-dependently. In particular, intracellular accumulation of [^{3}H]ipratropium in HEK293/OCTN2 cells in 30 min was 7.8-fold greater than that of MOCK cells, indicating that ipratropium is a preferred substrate for

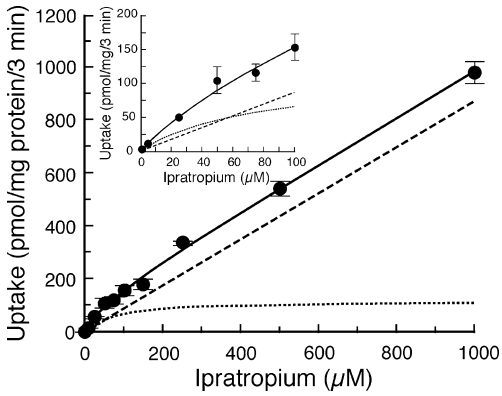

Figure 3. Concentration dependence of [^{3}H]ipratropium uptake by BEAS-2B cells. Total (solid line), saturable (dotted line), and nonsaturable (broken line) uptake of ipratropium by BEAS-2B cells was measured at 37 °C for 3 min. Each line represents the uptake calculated by using the kinetic parameters K_m, V_{max} and k_d as described in Results. The inset shows the results for ipratropium uptake rates up to 100 μM.

Table 2. Inhibitory Effect of Various Organic Cations on Ipratropium Uptake by BEAS-2B Cells[a]

inhibitor	concn (mM)	rel uptake (% of control)
control		100.0 ± 0.95
TEA	0.1	85.1 ± 2.95*
	1	73.2 ± 4.78*
quinidine	0.1	50.9 ± 11.36*
	1	33.5 ± 12.33*
corticosterone	0.025	75.9 ± 4.44*
	0.2	68.9 ± 8.06*
ergothioneine	0.2	78.3 ± 4.31*
	1	72.4 ± 4.10*
carnitine	0.2	85.4 ± 8.10
	1	78.3 ± 2.89*

[a] Uptake of [^{3}H]ipratropium (15 nM) by BEAS-2B cells was measured over 3 min at pH 7.4 at 37 °C in the absence (control) and the presence of organic cations and zwitterions. Data are shown as mean ± SEM ($n = 4$). *$p < 0.05$ by Student's t test.

OCTN2 (Figure 4A,B). Saturation kinetics of ipratropium uptake is shown in Figure 5A,B. Since each Eadie−Hofstee plot gave a single straight line, a single saturable component for ipratropium transport was detected, suggesting that no other transporters were involved kinetically (Figure 5 inset). The kinetic parameters, K_m and V_{max}, of OCTN1 and OCTN2 were 444 ± 108 μM and 238 ± 28.6 pmol/mg protein/5 min (OCTN1), and 53.0 ± 1.27 μM and 76.0 ± 1.15 pmol/mg protein/30 s (OCTN2), respectively. In addition, uptake of [^{3}H]tiotropium by HEK293/OCTN1 and HEK293/OCTN2 cells was similarly examine and compared to that by MOCK cells. The uptake for 30 min was 1.6- and 2.2-fold greater than that by MOCK cells (data not shown), demonstrating that tiotropium is a substrate for OCTN1 and OCTN2, though the intracellular accumulation of tiotropium by HEK293/OCTN2 cells was lower than that of ipratropium.

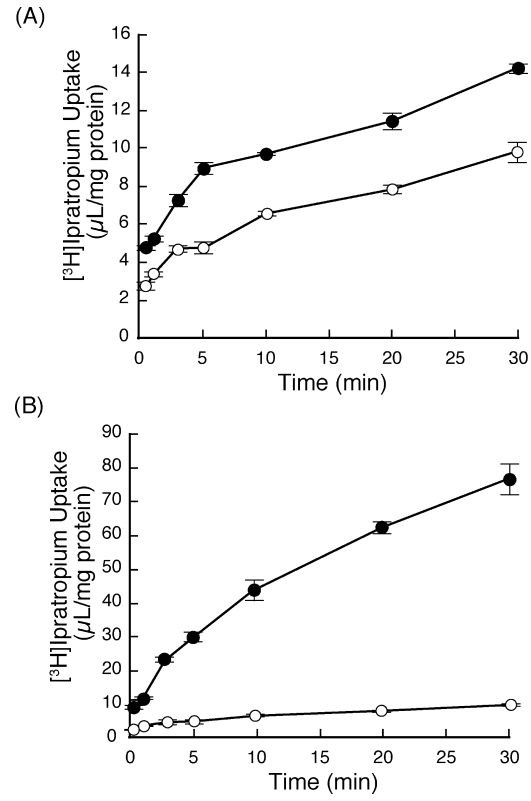

Figure 4. Time course of [^{3}H]ipratropium uptake by OCTN1- or OCTN2-expressing HEK293 cells. Uptake of [^{3}H]ipratropium (15 nM) by HEK293/ OCTN1 (A) or HEK293/OCTN2 (B) (closed circle) and pcDNA 3.1 vector-transfected cells (open circle, MOCK) was measured at pH 7.4 and 37 °C. Data are shown as mean ± SEM ($n = 3$).

Characterization of Ipratropium Uptake by OCTN1- and OCTN2-Expressing HEK293 Cells. The inhibitory effects of quinidine, TEA, ergothioneine and/or carnitine on ipratropium uptake by OCTN1- and OCTN2-expressing HEK293 cells are shown in Figure 6A, 6B, respectively. [^{3}H]Ipratropium uptake by OCTN1-expressing HEK293 cells was significantly reduced to 12.0% of control in the presence of quinidine, while the inhibitory effects of TEA and ergothioneine were marginal (Figure 6A). In the case of OCTN2, all three compounds at the concentration of 1 mM significantly reduced [^{3}H]ipratropium uptake. As a percentage of the uninhibited control, the order of inhibitory effects was quinidine (6.2%) > TEA (41.5%) > carnitine (54.7%) (Figure 6B), which is the same order as that of inhibition of ipratropium uptake by BEAS-2B cells (Table 2). Ipratropium also inhibited the uptake of ergothioneine by OCTN1-expressing HEK293 cells and carnitine by OCTN2-expressing HEK293 cells (Figure 6C, 6D, respectively), although the degree of inhibition at the maximum concentration tested (1 mM) was insufficient to allow calculation of accurate IC_{50} values.

When extracellular Na^+ was replaced with *N*-methyl-D-glucamine at equimolar concentration, the uptake of iprat-

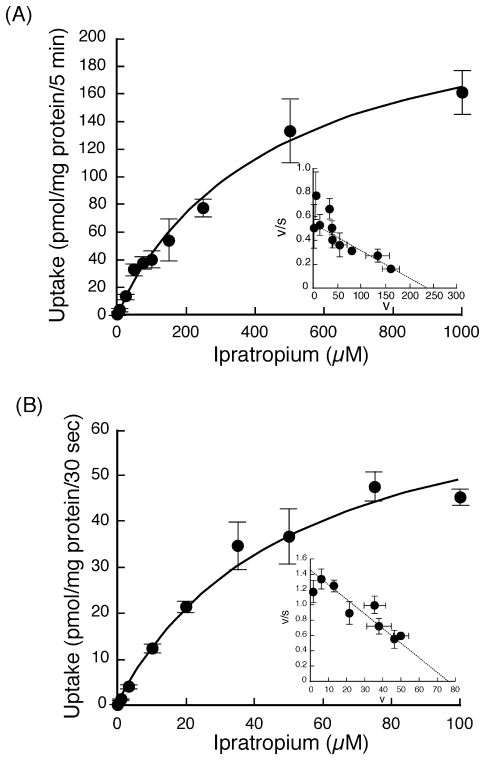

Figure 5. Concentration dependence of [^{3}H]ipratropium uptake by HEK293/OCTN1 or HEK293/OCTN2 cells. Uptake of ipratropium by HEK293/OCTN1 (A) or HEK293/OCTN2 (B) cells was measured at pH 7.4 and 37 °C for 5 min or 30 s, and is shown as the value after subtraction of the corresponding MOCK uptake. The solid line represents the uptake calculated by using the kinetic parameters K_m and V_{max} as described in Results. The inset shows an Eadie−Hofstee plot of saturable uptake of ipratropium. Data are shown as mean ± SEM ($n = 4$).

ropium was not affected (Figure 6E). Under the same conditions, uptake of ergothioneine and of carnitine was completely abolished (data not shown).

Contributions of OCTN1 and OCTN2 to Ipratropium Uptake in BEAS-2B Cells. To determine the contributions of OCTNs to ipratropium uptake by BEAS-2B cells, knock-down studies were conducted by transfection with shRNA-expression vectors. The levels of mRNA expression of OCTN1 and OCTN2 in BEAS-2B cells transfected with shRNA-expression vectors flanked by miR sequence were significantly decreased by 60.4% and 60.6%, respectively, compared with those in cells transfected with a scrambled sequence as a negative control (Table 3). Transport activity of OCTN1 and OCTN2 measured using a reference substrate [^{3}H]ergothioneine and [^{3}H]carnitine decreased by 22.9% and 46.6%, convincing the silencing of OCTN1 and OCTN2, respectively (Table 3). [^{3}H]Ipratropium uptake by BEAS-2B cells in which OCTN2 was silenced was significantly decreased to 14.8 ± 6.0% of control cells, whereas [^{3}H]ipratropium uptake by cells in which OCTN1 was silenced was decreased by only 21.8% (statistically insignificant *$p >$ 0.551, Figure 7). These data clearly suggest that OCTN2 is the predominant contributor to ipratropium uptake in BEAS-2B cells.

Discussion

In the present study, we explored the transport mechanism of the anti-COPD drug, ipratropium bromide, in human airway epithelial BEAS-2B cells. Our results indicate that OCTN2 is the predominant contributor to ipratropium uptake by bronchial derived cells.

As shown in Figure 1, the expression profile of organic cation transporters determined by RT-PCR in BEAS-2B cells was in good agreement with the previous reports, showing abundant expression of OCTN1 and OCTN2, but little or no expression of OCT1, OCT2 and OCT3 in human airway epithelial cells in air−liquid interface culture[9] and human bronchial epithelial 16HBE14o- cells.[22]

Prior to the work presented here, only salbutamol, a cationic β_2-adrenergic receptor antagonist used to treat COPD, has been shown to be actively absorbed from human airway epithelium,[9,23] though the transporter protein involved has not yet been identified. Since there are similarities between salbutamol and ipratropium in physicochemical properties, such as lipophilicity and membrane permeability, salbutamol may share the same transporter with ipratropium.[16] Here, we have demonstrated for the first time that OCTN1 and OCTN2 mediate uptake of the cationic anticholinergic drug ipratropium in human airway epithelial BEAS-2B cells. In addition, when OCTN2 expression was knocked-down in BEAS-2B cells, we observed a significant decrease of ipratropium uptake. Furthermore, we observed an increased uptake of tiotropium by HEK293/OCTN1 and HEK293/OCTN2 cells, compared to that by MOCK cells (data not shown). Thus, the present work provides the first evidence that OCTN2 contributes at least in part to the delivery of cationic anti-COPD drugs across pulmonary airway epithelium. Although other members of OCT family are not expressed in BEAS-2B cells, they appear to be weakly expressed at the mRNA level in another bronchial epithelial cell line, Calu-3.[22] Therefore, further study is warranted to clarify the possible contribution of these OCTs to the uptake of cationic anti-COPD drugs.

Functional characterization of OCTN2-mediated transport has revealed that OCTN2 is an Na^+-dependent, high-affinity transporter for carnitine, but alternatively functions as an

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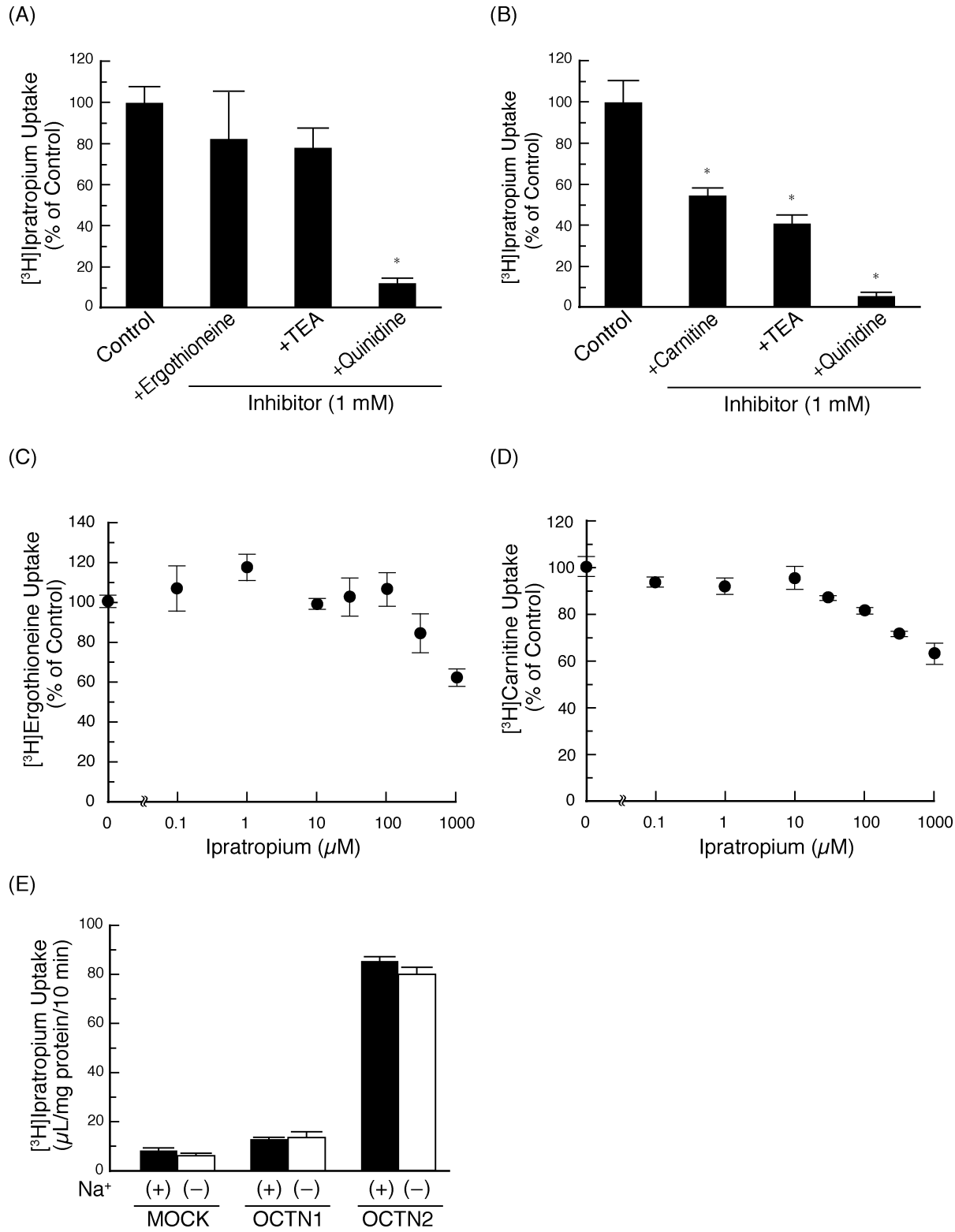

Figure 6. Characterization of ipratropium uptake by OCTN1- or OCTN2-expressing HEK293 cells. The effect of inhibitors on [^{3}H]ipratropium uptake by (A) OCTN1- or (B) OCTN2-expressing HEK 293 cells was measured at pH 7.4 and 37 °C for 5 min or 30 s, respectively. The inhibitory effect of ipratropium on (C) [^{3}H]ergothioneine uptake by OCTN1, and (D) [^{3}H]carnitine uptake by OCTN2 was measured at pH 7.4 and 37 °C for 30 s. (E) Sodium-dependence of [^{3}H]ipratropium uptake by OCTN1- or OCTN2-expressing HEK293 cells was measured at pH 7.4 and 37 °C for 10 min. Sodium chloride was replaced with an equal concentration of *N*-methylglucamine chloride. All data are shown as the values after subtraction of the corresponding MOCK uptake. Data are shown as mean ± SEM ($n = 4$), and an asterisk (*) indicates a significant difference from the control (cells transfected with a scrambled shRNA) by Student's *t* test ($p < 0.05$).

Table 3. Ergothioneine and Carnitine Uptake by BEAS-2B Cells with OCTN1 or OCTN2 Being Silenced[a]

	rel mRNA expression (% of scramble)		rel uptake (% of scramble)	
siRNA	OCTN1	OCTN2	[^{3}H]ergothioneine	[^{3}H]carnitine
control	100.0 ± 9.47	100.0 ± 6.46	100.0 ± 7.34	100.0 ± 15.0
OCTN1	39.6 ± 13.9*	104.7 ± 20.6	77.1 ± 1.06*	104.2 ± 9.41
OCTN2	88.5 ± 3.89	39.4 ± 9.47*	93.7 ± 12.0	53.4 ± 1.94*

[a] mRNA expression of OCTN1 and OCTN2 in BEAS-2B cells expressing shRNA to OCTN1 and OCTN2 were quantified as described in the Experimental Section, respectively, and then normalized by that in BEAS-2B cells transfected with the pcDNA6.2-GW/EmGFP-miR-negative control plasmid containing scramble oligonucleotide not related to OCTNs (control). Uptake of [^{3}H]ergothioneine (2 μM) and of [^{3}H]carnitine (0.1 μM) by OCTN1- and OCTN2-knocking-down BEAS-2B cells was measured for 3 min at 37 °C, respectively, and normalized by that in the control. Data are shown as mean ± SEM (n = 3), and an asterisk (*) indicates a significant difference from the control ($p < 0.05$ by Student's t test).

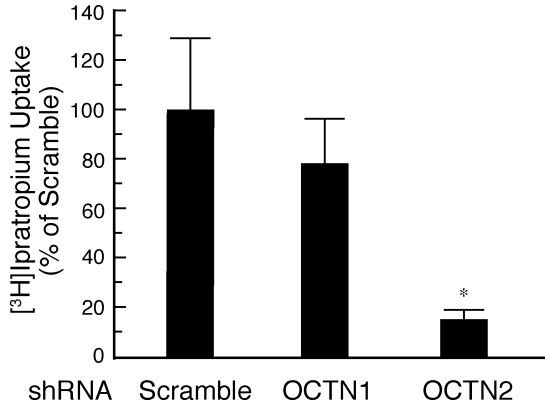

Figure 7. Effects of RNA interference on [^{3}H]ipratropium uptake by BEAS-2B cells. Uptake of [^{3}H]ipratropium (15 nM) by shRNA-transfected BEAS-2B cells was measured at 37 °C, and data are shown as the values after subtraction of the uptake at 4 °C. Data are shown as mean ± SEM (n = 4), and an asterisk (*) indicates a significant difference from the control (cells transfected with a scrambled shRNA) by Student's t test ($p < 0.05$).

Na^+-independent polyspecific cation transporter.[2,6,8,19,24] OCTN2 prefers carnitine to other organic cationic compounds, such as TEA; the K_m value of carnitine (4.34 μM) is much lower than that of TEA (304 μM).[2,25] Our data revealed that the affinity of ipratropium for OCTN2 was relatively low, with a K_m of 53 μM (Figure 5). We reported mutual inhibition between carnitine and organic cations in OCTN2-mediated transport, demonstrating that OCTN2 possesses at least two distinct binding sites for substrates.[25] As shown in Table 2 and Figure 6B, ipratropium uptake by BEAS-2B and HEK293/OCTN2 cells was most potently inhibited by quinidine among the compounds tested, although the binding affinity of quinidine to OCTN2 is much lower than that of carnitine.[2,25] This could be because ipratropium shares the same binding site with the cation quinidine, but not that of zwitterion-like carnitine. This notion is supported by the results of a mutual inhibition study (Figure 6D) and observation of Na^+-independent OCTN2-mediated ipratropium uptake (Figure 6E) by HEK293/OCTN2 cells. Our data also suggest that OCTN1-mediated ipratropium uptake could occur in a similar manner to OCTN2 (Figure 6A,C,E).

In clinical application, 20 μg of ipratropium bromide is the typically delivered single inhaled dose. Since the volume of fluid available in the airway surface was estimated to be approximately 1 mL in the first 12 generations of the airways,[26] assuming the entire dispensed dose is delivered to the lung, the concentration of ipratropium in the tissue could reach up to 50 μM. This concentration is comparable to the estimated K_m values in BEAS-2B (K_m of 78 μM, Figure 3) and HEK293/OCTN2 cells (53 μM, Figure 5). Hence, OCTN2 is unlikely to be saturated in the *in vivo* airway lumen after inhalant administration. Besides, OCTN2 was indicated to be the major contributor to carrier-mediated uptake of ipratropium in BEAS-2B cells (Figure 7). These results indicate a potential role for OCTN2-mediated transport of ipratropium, and possibly tiotropium, in facilitating their pharmacological bronchodilatory effects, and inhaled systemic availability, despite the relative hydrophilicity and poor membrane permeability of these drugs.[16]

Once ipratropium bromide is absorbed, this drug distributes widely, and a significant proportion is rapidly excreted via the kidneys. Renal clearance of ipratropium is about six times larger than the glomerular filtration rate,[14] indicating active tubular secretion. Since OCTN2 is also highly expressed in the apical membrane of proximal tubular cells, such rapid renal secretion might be mediated by OCTN2 expressed in this organ. Although little is known about clinical drug−drug interaction in renal excretion of this drug, inhibition of this clearance mechanism by a coadministered OCTN inhibitor might reasonably result in unexpectedly high circulating concentrations of the drug (increased C_{max} and/or increased plasma $t_{1/2}$), possibly leading to enhanced toxicity.[27] Furthermore, since OCTN2 is also expressed in the human

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heart,[28] it may facilitate *in vivo* tissue distribution of ipratropium, potentially mediating cardiovascular side effects.

In conclusion, ipratropium and tiotropium were identified as drug substrates of OCTN1 and OCTN2. Our results indicate that OCTN2 is the predominant contributor to carrier-mediated uptake of ipratropium by BEAS-2B cells. Further studies are necessary to establish whether or not other transporters expressed in the human respiratory epithelial barrier are involved in the transport of cationic compounds, including anti-COPD drugs. Finally, our findings indicate that drug delivery via the pulmonary route, making use of the carrier-mediated mechanisms that we have uncovered, may be an attractive candidate for achieving systemic exposure.

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