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# The human OCTN1 (SLC22A4) reconstituted in liposomes catalyzes acetylcholine transport which is defective in the mutant L503F associated to the Crohn's disease

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ABSTRACT

The organic cation transporter (OCTN1) plays key roles in transport of selected organic cations, but understanding of its biological functions remains limited by restricted knowledge of its substrate targets. Here we show capacity of human OCTN1-reconstituted proteoliposomes to mediate uptake and efflux of [$^3$H]acetylcholine, the Km of transport being 1.0 mM with $V_{max}$ of 160 nmol·mg$^{-1}$ protein·min$^{-1}$. OCTN1-mediated transport of this neurotransmitter was time-dependent and was stimulated by intraliposomal ATP. The transporter operates as uniporter but translocates acetylcholine in both directions. [$^3$H]acetylcholine uptake was competitively inhibited by tetraethylammonium, γ-butyrobetaine and acetylcarnitine, and was also inhibited by various polyamines. Decreasing intraliposomal ATP concentrations increased OCTN Km for acetylcholine, but $V_{max}$ was unaffected. Evaluation of the acetylcholine transporter properties of a variant form of OCTN1, the Crohn's disease-associated 503F variant, revealed time course, Km and $V_{max}$ for acetylcholine uptake to be comparable to that of wild-type OCTN1. Km for acetylcholine efflux was also comparable for both OCTN1 species, but $V_{max}$ of OCTN1 503F-mediated acetylcholine efflux (1.9 nmol·mg$^{-1}$ protein·min$^{-1}$) was significantly lower than that of wild-type OCTN1 (14 nmol·mg$^{-1}$ protein·min$^{-1}$). These data identify a new transport role for OCTN1 and raise the possibility that its involvement in the non-neuronal acetylcholine system may be relevant to the pathogenesis of Crohn's disease.



## 1. Introduction

The OCTN1 protein belongs to a small family of transporter proteins – the organic cation transporters – which share more than 66% identity with one another [1]. These proteins originated after the divergence of vertebrates and invertebrates [2]. Definition of OCTN1 substrates began many years ago [1,3–6], but has been greatly enhanced recently by capacity to reconstitute in proteoliposomes the human OCTN1 overexpressed in bacteria [7,8]. The transporter has been inserted into the proteoliposomal membrane with the same orientation as in the cell membrane. Via use of this experimental system, tetraethylammonium (TEA), has been identified as very good substrate for OCTN1 transporter. In this system, OCTN1 has been shown to transport TEA with eight fold more efficiency than some of its other previously-identified substrates such as ergothioneine [5] and carnitine [6]. The data performed with the reconstituted transporter diverge from some data reported in intact cells, however, they are consistent with other data performed with intact cells [1,3,4]. Availability of reconstituted hOCTN1 has also enabled better definition of other aspects of its transport properties, revealing its interaction with not only known substrates such as tetraethylammonium and acetylcarnitine, but also with a number of new species, including the neurotransmitter acetylcholine. While best recognized for its neurotransmitter functions, acetylcholine has recently emerged as an important player in the non-neuronal cholinergic system [9,10]. This system includes the airways, alimentary tract, skin, placenta, heart, skeletal muscle, urogenital tract and other tissues now known to express choline acetyltransferase as well as acetylcholine receptor and degrading enzyme, cholinesterase [9]. Within these tissues, roles for acetylcholine in cell proliferation and differentiation, cytoskeletal organization, cell–cell contact and even inflammation are now appreciated [9]. Acetylcholine involvement in these processes requires its transport into or efflux from the relevant cells. However, differently from the nervous tissues in which a transporter for acetylcholine has been described [11], until now, the mechanisms for acetylcholine trafficking in non-nervous system cells have remained unknown. Recent data, however, suggesting that OCTN1 [8]

*Abbreviations:* TEA, tetraethylammonium; PLP, pyridoxal 5-phosphate; ANTP, adenosine 5′-(β,γ-imido)triphosphate; MTSET, sodium (2-(trimethylammonium)ethyl methanethiosulfonate); p-OHMB, p-hydroxymercuribenzoate; NEM, N-ethylmaleimide

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binds acetylcholine, raises the possibility that OCTN1 also serves as an acetylcholine transporter as previously found also for members of the OCT transporter family which mediate acetylcholine transport in placenta [9] and other tissues [12,13]. By assessing effects of hOCTN1 reconstituted into liposomes, on acetylcholine transport, we demonstrate here that OCTN1 modulates uptake and efflux of acetylcholine and that its capacity to transport this molecule is altered by the L503F substitution associated with inflammatory bowel disease [14].

## 2. Materials and methods

### 2.1. Materials

Amberlite XAD-4, egg yolk phospholipids (3-sn-phosphatidylcoline from egg yolk) and Triton-X100 were purchased from Fluka; acetylcholine iodide [acetyl-$^3$H] from Perkin-Elmer, Sephadex G-75, L-carnitine, tetraethylammonium chloride from Sigma, ergothioneine from Santa Cruz Biotechnology. All the other reagents were of analytical grade.

### 2.2. Reconstitution of the hOCTN1 transporter into liposomes

hOCTN1 and hOCTN1 503F were over-expressed in *E.coli* and purified as previously described [7] with the exception that the NaCl concentration in the buffer used for the elution of the purified protein fraction, was 50 mM instead of 200 mM. This resulted in an increase of the transport activity of the hOCTN1 with respect to the previous procedure [8] with no variations in the amount and purity of the protein. The purified hOCTN1 and hOCTN1 503F were reconstituted by removing the detergent from mixed micelles containing detergent, protein and phospholipids by incubation with Amberlite XAD-4 in a batch-wise procedure [8]. The composition of the initial mixture used for reconstitution (except when differently indicated) was: 180 μl of the purified protein (6 μg protein in 0.1% Triton-X100), 120 μl of 10% Triton X-100, 120 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [15], 16 mM ATP di-sodium salt buffered at pH 8.0, 10 mM Tris/HCl (pH 8.0) in a final volume of 700 μl. After vortexing, this mixture was incubated with 0.55 g Amberlite XAD-4 under rotatory stirring (1400 rpm) at room temperature for 45 min.

### 2.3. Transport measurements

Proteoliposomes (550 μl) were passed through a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 10 mM Tris/HCl (pH 8.0). Proteoliposomes (550 μl) were collected from these columns and divided in aliquots (samples) of 100 μl. Transport was started by adding the indicated concentrations of [$^3$H]acetylcholine to the proteoliposome samples, as indicated in the figure legends and stopped by adding 2 mM PLP at the desired time interval. In control samples the inhibitor was added at time zero according to the inhibitor stop method [16]. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-75 column (0.6 cm diameter × 8 cm height) in order to separate the external from the internal radioactivity. Liposomes were eluted with 1 ml 50 mM NaCl and collected in 4 ml of scintillation mixture, vortexed and counted. The experimental values were corrected by subtracting the respective control. The PLP insensitive radioactivity associated to the control samples was always less than 15% with respect to the PLP sensitive acetylcholine transport. The initial rate of transport expressed as nmol · mg$^{-1}$ protein · min$^{-1}$, was measured by stopping the reaction after 5 min, i.e., within the initial linear range of [$^3$H]acetylcholine uptake into the proteoliposomes (see Fig. 1). For efflux measurements, aliquots of the same pool of proteoliposomes were incubated with external [$^3$H]acetylcholine at different concentrations. After 90 min, corresponding to the optimal intraliposomal [$^3$H]acetylcholine accumulation the proteoliposomes were passed again trough a Sephadex G-75 column (0.7 cm diameter × 15 cm height) preequilibrated with 10 mM Tris/HCl (pH 8.0) and the time course of [$^3$H]acetylcholine efflux was then measured as described for the uptake procedure. The efflux reaction was stopped at the indicated time intervals by adding 2 mM PLP. The uptake time course data were interpolated using a first order rate equation from which rate constants were derived as the product of k (the first order rate constant) and the transport at the equilibrium. The efflux time course data were interpolated using a single exponential decay equation from which the initial rates of the efflux were calculated as the product of k (the first order rate constant) and the amount of [$^3$H] acetylcholine effluxed at infinite time (equilibrium). The concentration of internal [$^3$H]acetylcholine at the beginning of the efflux procedure was equal to the initial external [$^3$H]acetylcholine concentration.

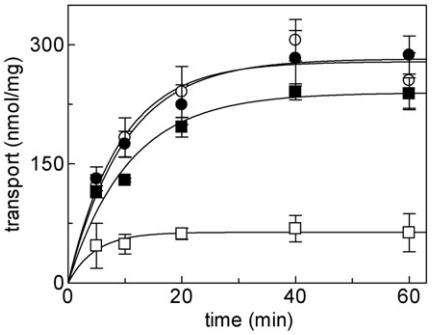


**Fig. 1.** Time course of acetylcholine uptake by reconstituted proteoliposomes. The reconstitution was performed as described in Materials and methods. Transport was started adding 0.1 mM [$^3$H]acetylcholine at time zero to proteoliposomes (●,■,○) or proteoliposomes reconstituted with protein treated for 20 min at 100 °C (□). In (■) 2 mM acetylcholine was present inside the proteoliposomes. In (●) the transport reaction was stopped at the indicated times and the specific transport activity was calculated according to the inhibitor stop-method. In (□, ■,○), the transport reaction was stopped at the indicated times by directly passing the proteoliposomes through Sephadex G-75 columns as described in Materials and methods and the specific transport activity was calculated as the difference between the uptake in the presence and absence of incorporated protein (not reported). The values are means ± S.D. from three experiments.

Kinetic constants were estimated using non linear fitting of the experimental data in the Michaelis–Menten equation. Lineweaver–Burk plots were used for presentation of inhibition. The Grafit (version 5.0.3) software was used for calculations.

### 2.4. Other methods

Protein amount was measured by densitometry of Coomassie-stained protein bands carried out using the Chemidoc imaging system equipped with Quantity One software (Bio-Rad), as previously described [17]. Sequence motifs were predicted by the Prosite tool.

## 3. Results

### 3.1. hOCTN1 mediates acetylcholine transport

Uptake of [$^3$H]acetylcholine in hOCTN1-reconstituted proteoliposomes was initially measured as a function of time (Fig. 1), with PLP used to terminate transport. This analysis revealed acetylcholine uptake to be significantly higher in hOCTN1 compared to "empty" (no hOCTN1-containing; control) liposomes (not shown) with the rate of transport being 30 ± 7.8 nmol·mg$^{-1}$ protein·min$^{-1}$. These values appear to accurately reflect specific acetylcholine transport activity as the observed time course of transport activity mirrored that calculated by subtracting the control values. To gain insights into mode of transport, [$^3$H]acetylcholine uptake into acetylcholine-loaded hOCTN1-containing liposomes was also evaluated. As this analysis revealed specific transport to be similar or slightly lower in the presence compared to absence of internal substrate, it appears that the transporter catalyzes uniport rather than antiport. Comparison

of the acetylcholine transporter activity of boiled versus native hOCTN1 in this system revealed much lower transport of the boiled protein, indicating that hOCTN1 activity is required for its transport of acetylcholine. Transport activity was also increased by raising pH from 6.0 (activity near zero) to 7.5 and remained nearly constant under pH conditions of 7.5–8.5 (not shown).

It was previously hypothesized in experiments performed with cells that ATP may stimulate hOCTN1 transport activity [18]. Indeed, TEA uptake catalyzed by reconstituted hOCTN1 was stimulated by intraliposomal ATP [8]. Thus the effect of ATP on hOCTN1-mediated acetylcholine transport was evaluated. For this purpose the transport assays were performed in proteoliposomes reconstituted with varying concentrations of ATP (Fig. 2). A biphasic pattern of transport was observed, with activity plateauing initially at 6 mM ATP and again at 12 mM ATP. This effect, which was also previously reported in relation to TEA transport, was not due to ATP hydrolysis, as the same pattern was observed in tests using non-hydrolysable ATP analog (ANTP) rather than ATP (data not shown). Moreover, the activation by intraliposomal ATP was rather specific since internal adenosine, AMP and cAMP did not exert appreciable effects and no variations of transport activity respect to the condition with zero internal ATP were observed in the presence of internal NaCl or sucrose at concentrations ranging from 5 to 50 mM (not shown). Differently from the intraliposomal side, ATP externally added did not exert any activation effect (Fig. 2).

To explore the effects of other compounds on this transport pathway, the hOCTN1-reconstituted liposomes were next assayed for capacity to transport acetylcholine in the presence of other known or putative OCTN1 substrates or inhibitors. Among the substrates tested, carnitine, betaine, ergothioneine, glucose, creatinine, creatine and GABA had no effect on acetylcholine transport when used at concentrations up to 1 mM (Table 1). By contrast, acetylcholine transport was almost completely inhibited by spermine and spermidine at concentrations as low as 0.5 mM and was also significantly suppressed by choline, acetylcarnitine, γ-butyrobetaine, tetraethylammonium and tetramethylammonium. Similarly, $HgCl_2$, MTSET and pOHMB, compounds known to interact with SH residues, as well as PLP, which interacts with $NH_2$ residues, all inhibited the transporter to varying degrees (Table 2 and Fig. 3). These inhibitory effects appeared specific to individual compounds, as transport activity was not affected by two other reagents, NEM and mersalyl, which also recognize SH residues. The effects of PLP, the inhibitor used in most of our transport assays, were concentration-dependent, acetylcholine transport being entirely abrogated at PLP concentration of 1.5 mM and $IC_{50}$ being 0.39 ± 0.051 mM (Fig. 3).

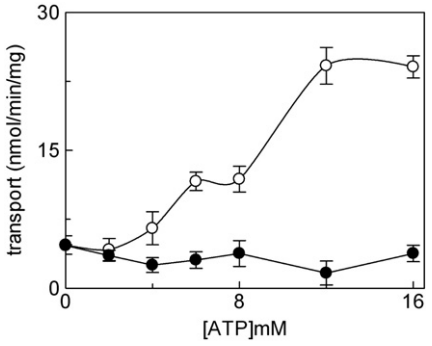


**Fig. 2.** Dependence of acetylcholine transport on the concentration of intra- and extraliposomal ATP. The reconstitution was performed as described in Materials and methods except that the indicated concentrations of ATP were added to the reconstitution mixture (○) or to the external side of proteoliposomes without internal ATP (●). Transport was started adding 0.1 mM [$^3$H]acetylcholine to proteoliposomes. The values are means ± S.D. from three experiments.

**Table 1**
Effect of different substrates on the reconstituted hOCTN1. Transport was measured as 0.1 mM [$^3$H]acetylcholine uptake into proteoliposomes, reconstituted as described in Materials and methods, in 10 min. The molecules were added 1 min before the labeled substrate at the indicated concentrations. Percent residual activity was calculated for each experiment with respect to the control sample (referred as 100%). The results are means ± S.D. of the percentage of three experiments.

| Substrate | Concentration (mM) | Residual activity (%) |
|---|---|---|
| acetylcarnitine | 0.5 | 46 ± 15[a] |
| | 1.0 | 24 ± 0.9[a] |
| carnitine | 0.5 | 90 ± 2.6[a] |
| | 1.0 | 80 ± 11 |
| tetraethylammonium | 0.5 | 66 ± 9.8[a] |
| | 1.0 | 49 ± 5.3[a] |
| choline | 0.5 | 60 ± 2.8[a] |
| | 1.0 | 40 ± 6.9[a] |
| betaine | 0.5 | 86 ± 10 |
| | 1.0 | 83 ± 2.3[a] |
| ergothioneine | 0.5 | 90 ± 4.9 |
| | 1.0 | 67 ± 3.2[a] |
| tetramethylammonium | 0.5 | 54 ± 8.3[a] |
| | 1.0 | 44 ± 2.9[a] |
| glucose | 0.5 | 80 ± 24 |
| | 1.0 | 80 ± 3.5[a] |
| creatinine | 0.5 | 75 ± 0.4[a] |
| | 1.0 | 74 ± 10 |
| creatine | 0.5 | 94 ± 1.9 |
| | 1.0 | 78 ± 4.1[a] |
| γ-aminobutyric acid | 0.5 | 78 ± 6.6[a] |
| | 1.0 | 63 ± 1.2[a] |
| γ-butyrobetaine | 0.5 | 45 ± 5.1[a] |
| | 1.0 | 27 ± 6.3[a] |
| spermine | 0.5 | 6.8 ± 1.5[a] |
| spermidine | 0.5 | 7.4 ± 3.5[a] |

[a] Significantly different from the control (100%) as estimated by Student's t test ($P<0.05$).

### 3.2. Kinetics of hOCTN1-mediated acetylcholine transport

The kinetics of hOCTN1-mediated acetylcholine transport were also explored, these assays measuring uptake of varying concentrations of [$^3$H]acetylcholine by the hOCTN1-reconstituted liposomes. This analysis revealed the half saturation constant (Km) to be 1.0 ± 0.21 mM and $V_{max}$ to be 160 ± 32.0 nmol·mg$^{-1}$ protein·min$^{-1}$. Similar studies were also carried out in the context of added substrates or inhibitors so as to delineate the extent to which these compounds modulate the kinetics of acetylcholine transport. As shown in Fig. 4, these analyses revealed competitive inhibition of acetylcholine transport by TEA, γ-butyrobetaine and acetylcarnitine and mixed inhibition by spermidine and spermine. The kinetic constants are summarized in Table 3. ATP effects on acetylcholine kinetics were also assessed measuring the

**Table 2**
Effect of inhibitors on the reconstituted hOCTN1. Transport was measured as 0.1 mM [$^3$H]acetylcholine uptake into proteoliposomes, reconstituted as described in Materials and methods, in 10 min. The molecules were added 1 min before the labeled substrate at the indicated concentrations. Percent residual activity was calculated for each experiment with respect to the control sample (referred as 100%). The results are means ± S.D. of the percentage of three experiments.

| Inhibitor | Concentration (mM) | Residual activity (%) |
|---|---|---|
| mersalyl | 0.1 | 128 ± 18 |
| | 1.0 | 84 ± 6.8 |
| NEM | 0.5 | 96.7 ± 11 |
| | 1.0 | 92.3 ± 0.9 |
| HgCl2 | 0.02 | 57 ± 3.9[a] |
| | 0.2 | 13 ± 0.3[a] |
| p-OHMB | 0.02 | 65 ± 6.7[a] |
| | 0.2 | 25 ± 12[a] |
| MTSET | 0.1 | 93 ± 4.1 |
| | 1.0 | 38 ± 3.2[a] |

[a] Significantly different from the control (100%) as estimated by Student's t test ($P<0.05$).

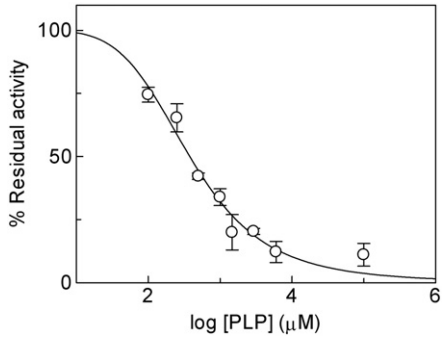


**Fig. 3.** Dose–response curves for the inhibition of the reconstituted transporter by PLP. Transport was measured by adding 0.1 mM [$^3$H]acetylcholine to proteoliposomes in the presence of PLP at the indicated concentrations. Percent residual activity with respect to the control is reported. The values are means ± S.D. from three experiments.

dependence of the transport rate on acetylcholine concentration in the presence of 16 mM internal ATP, i.e., the condition used for all the other experiments, or 4 mM ATP. At the lower ATP concentration the Km of OCTN1 for acetylcholine was higher (1.6 mM) than at 16 mM ATP, whereas the $V_{max}$ did not change (Fig. 5).

### 3.3. The hOCTN1 503F mutant impairs hOCTN1-mediated acetylcholine transport

In previous studies of hOCTN1 activity, a variant form of hOCTN1 (503F mutant) associated with predisposition to Crohn's disease has been shown to alter hOCTN1-mediated TEA transport [14]. The effects of this variant on hOCTN1 acetylcholine transporter activity were thus assayed using proteoliposomes reconstituted with hOCTN1 503 F. Results of a time course assay (Fig. 6) revealed rate of [$^3$H]acetylcholine uptake

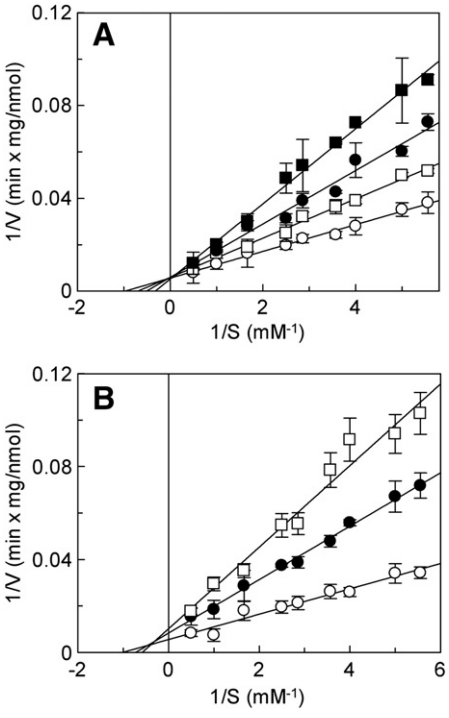


**Fig. 4.** Kinetic analysis of the inhibition of the reconstituted transporter by TEA, acetylcarnitine, γ-butyrobetaine and polyamines. The transport rate was measured, as described in Materials and methods adding [$^3$H]acetylcholine at the indicated concentrations to proteoliposomes in the absence (○) or presence of (A) 0.5 mM TEA (□), 0.4 mM acetylcarnitine (●) or 0.5 mM γ-butyrobetaine (■); (B) 40 μM spermidine (●) or 40 μM spermine (□). Data were plotted according to Lineweaver–Burk as reciprocal transport rate vs reciprocal acetylcholine concentration. The values are means ± S.D. from three experiments.

**Table 3**
Inhibition kinetic data of hOCTN1. Kinetic constants calculated from the experiments described in Fig. 4 A and B. Values are obtained by non linear regression analysis of means from at least three different experiments. For each data S.D. is reported which was obtained by non linear regression to each data set.

| Inhibitor | Km or Ki | Vmax (nmol·mg$^{-1}$·min$^{-1}$) |
|---|---|---|
| No inhibitor (Km for acetylcholine) | 1.0 ± 0.21 mM | 160 ± 32 |
| TEA | 1.3 ± 0.31 mM | |
| γ-butyrobetaine | 0.70 ± 0.32 mM | |
| Acetylcarnitine | 0.72 ± 0.25 mM | |
| Spermidine | 35 ± 7.1 μM | |
| Spermine | 18 ± 2.4 μM | |

mediated by the mutant hOCTN1 species to be similar to that of wild-type, the transport rate being 33 ± 5.2 nmol·mg$^{-1}$ protein·min$^{-1}$. As for wild-type, hOCTN1 503 F-mediated transport rate was affected by acetylcholine concentration, with Km being 0.47 ± 0.10 mM and $V_{max}$ = 177 ± 17 nmol·mg$^{-1}$ protein·min$^{-1}$ (Fig. 7). Capacity for transport in the opposite direction, i.e. efflux from the cell, was then assayed in proteoliposomes containing either wild-type or mutant hOCTN1. This assay confirmed capacity of wild-type hOCTN1 to mediate counter-transport of [$^3$H]acetylcholine, the residual radioactivity inside the proteoliposome providing a measure of the extent of efflux (Fig. 8). For wild-type hOCTN1, a rate of efflux of 3.6 ± 0.88 nmol·mg$^{-1}$ protein·min$^{-1}$ was observed, a value lower than the rate of acetylcholine uptake. Efflux of [$^3$H]acetylcholine from the hOCTN1 503 F-proteoliposomes was, however, even lower, the rate of transport being 0.41 ± 0.1 nmol·mg$^{-1}$ protein·min$^{-1}$. This apparent defect in driving [$^3$H]acetylcholine efflux was further studied using proteoliposome containing varying concentrations of [$^3$H]acetylcholine. As observed for substrate uptake, rates at which internal acetylcholine was transported outwards were concentration-dependent. The wild-type hOCTN1 exhibited a Km of 0.78 ± 0.14 mM and $V_{max}$ of 14 ± 2.0 nmol·mg$^{-1}$ protein·min$^{-1}$ (Fig. 9A); the hOCTN1 503 F species showed Km of 0.54 ± 0.10 mM and $V_{max}$ of 1.9 ± 0.40 nmol·mg$^{-1}$ protein·min$^{-1}$. Thus the variant manifests a significant impairment in capacity to transport acetylcholine substrate from the proteoliposomes, i.e. from the cell.

## 4. Discussion

The experimental model of reconstitution in liposomes allows to obtain reliable information on the function of transporters in absence of interferences by other transporters or enzymes [8,19–23]. Furthermore, the reconstitution is an essential tool for studying heterologously expressed human transporters which cannot be studied after extraction from tissues [8,24–27]. The functional properties of the reconstituted

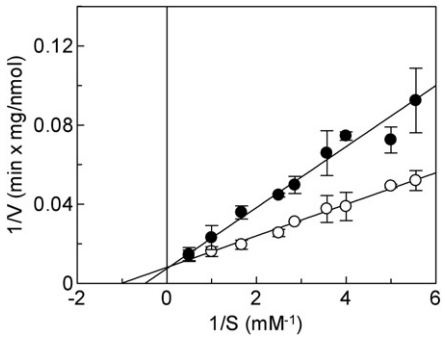


**Fig. 5.** Kinetic analysis of the acetylcholine transport in the presence of different intraliposomal ATP concentrations. The transport rate was measured, as described in Materials and methods adding [$^3$H]acetylcholine at the indicated concentrations to proteoliposomes containing 16 mM (○) or 4 mM (●) ATP. Data were plotted according to Lineweaver–Burk as reciprocal transport rate vs reciprocal acetylcholine concentration. The values are means ± S.D. from three experiments.

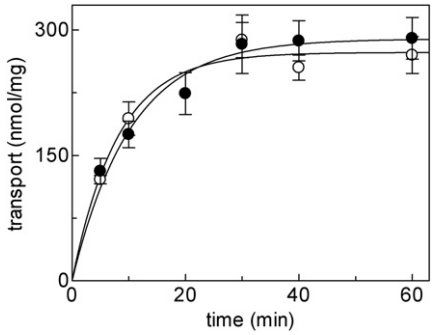


**Fig. 6.** Time course of acetylcholine uptake by proteoliposomes reconstituted with the L503F hOCTN1 in comparison with WT. The reconstitution was performed as described in Materials and methods. Transport was started adding 0.1 mM [$^3$H]acetylcholine at time zero to proteoliposomes. The transport reaction was stopped at the indicated times according to the inhibitor stop-method. Data from Fig. 1 concerning the WT (●) was also reported together with the mutant L503F (○). The values are means ± S.D. from three experiments.

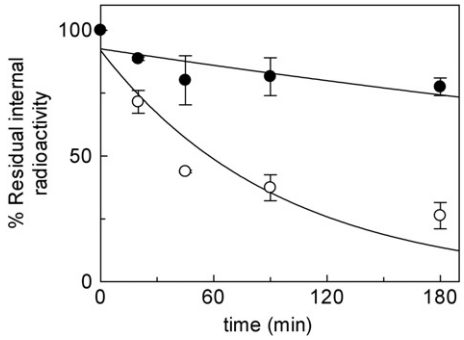


**Fig. 8.** Time course of acetylcholine efflux from proteoliposomes catalyzed by the reconstituted wild type and mutant transporters. [$^3$H]acetylcholine efflux was measured from proteoliposomes reconstituted with the wild type (○) or L503F mutant (●) after prelabelling by transporter-mediated exchange equilibration; the transport reaction was stopped at the indicated times as described in Materials and methods. The values are means ± S.D. from three experiments.

hOCTN1 strictly resembled the data on OCTN1 previously described by some authors in cells [1,3,4]. The reconstituted transporter seems to be in a native form, i.e., properly folded, since the kinetic constants for TEA are similar to those reported in transfected cells. However, some differences were found for ergothioneine respect to data obtained in cell systems, which may be due to different experimental conditions in the measurement of transport, i.e., lack of regulatory factors in proteoliposomes, exerting specific effects in cells on ergothioneine transport [8]. Concerning the side-specificity (intraliposomal) of the ATP effect (Fig. 2), this may be in agreement with the orientation of the transporter in the liposomal membrane as in the cell membrane [8]. The functional asymmetry of the transporter corresponds to the structural asymmetry; interestingly the analysis of the sequence of hOCTN1 reveals a nucleotide binding motif, which is intracellularly located as deduced by the hydropathy profile of the protein [7] (see also Fig. 10). However, all the experimental data concerning the OCTN1 function described so far [3–6], does not account for a specific physiological function of this transporter. Among the proposed OCTN1 substrates, TEA is a non physiological prototype cation, ergothioneine is an exogenous compound and carnitine was found by several authors to be transported at a very low rate [1, 3–6, 8]. The experimental data described in this work, shows that hOCTN1 catalyzes both uptake and efflux of acetylcholine. The Km for acethylcholine is very similar to that previously reported for the vesicular acetylcholine transporter VAchT [28], indicating that hOCTN1 should be suitable to mediate acetylcholine transport in vivo. The $V_{max}$ of hOCTN1 is comparable to that of another reconstituted human transporter, the organic cation transporter MATE 2 [27], which mediates TEA transport as the hOCTN1. The similar Km on the external and internal membrane side of hOCTN1, indicated that external and internal binding sites may be assembled by common amino acid residues, alternatively exposed outside or inside, depending on the conformational state (outwardly or inwardly directed) of the transporter. The lower $V_{max}$ found in the outward transport direction with respect to the inward may be explained by different activation energies associated with the conformational changes required for the two opposed transport processes or, alternatively, the two processes might be differently modulated by effectors, such as ATP. Kinetic analysis revealed that ATP slightly modulates the Km, i.e., the substrate–transporter interaction not the intrinsic transport activity ($V_{max}$), indicating that the nucleotide influences the

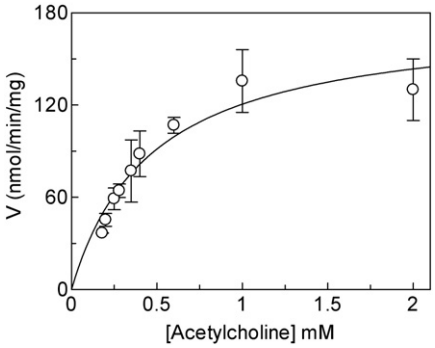


**Fig. 7.** Kinetic analysis of the acetylcholine transport catalyzed by the L503F hOCTN1. The transport rate was measured, as described in Materials and methods adding [$^3$H]acetylcholine at the indicated concentrations to proteoliposomes reconstituted with the L503F hOCTN1. Data were plotted according to Michaelis–Menten as transport rate vs acetylcholine concentration. The values are means ± S.D. from three experiments.

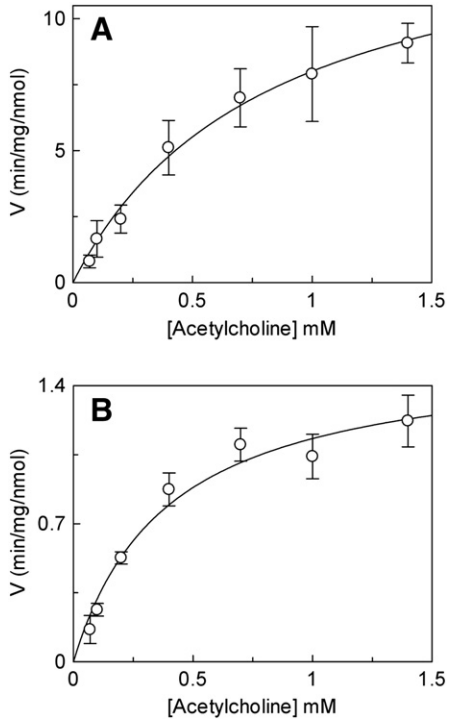


**Fig. 9.** Kinetic analysis of the acetylcholine efflux catalyzed by the reconstituted wild type and mutant transporters. [$^3$H]acetylcholine efflux rate was measured from proteoliposomes reconstituted with the wild type (A) or L503F mutant (B) after prelabelling by transporter-mediated exchange equilibration with different [$^3$H]acetylcholine concentrations, as described in Materials and methods. Data were plotted according to Michaelis–Menten as transport rate vs intraliposomal acetylcholine concentration (mM). The values are means ± S.D. from three experiments.

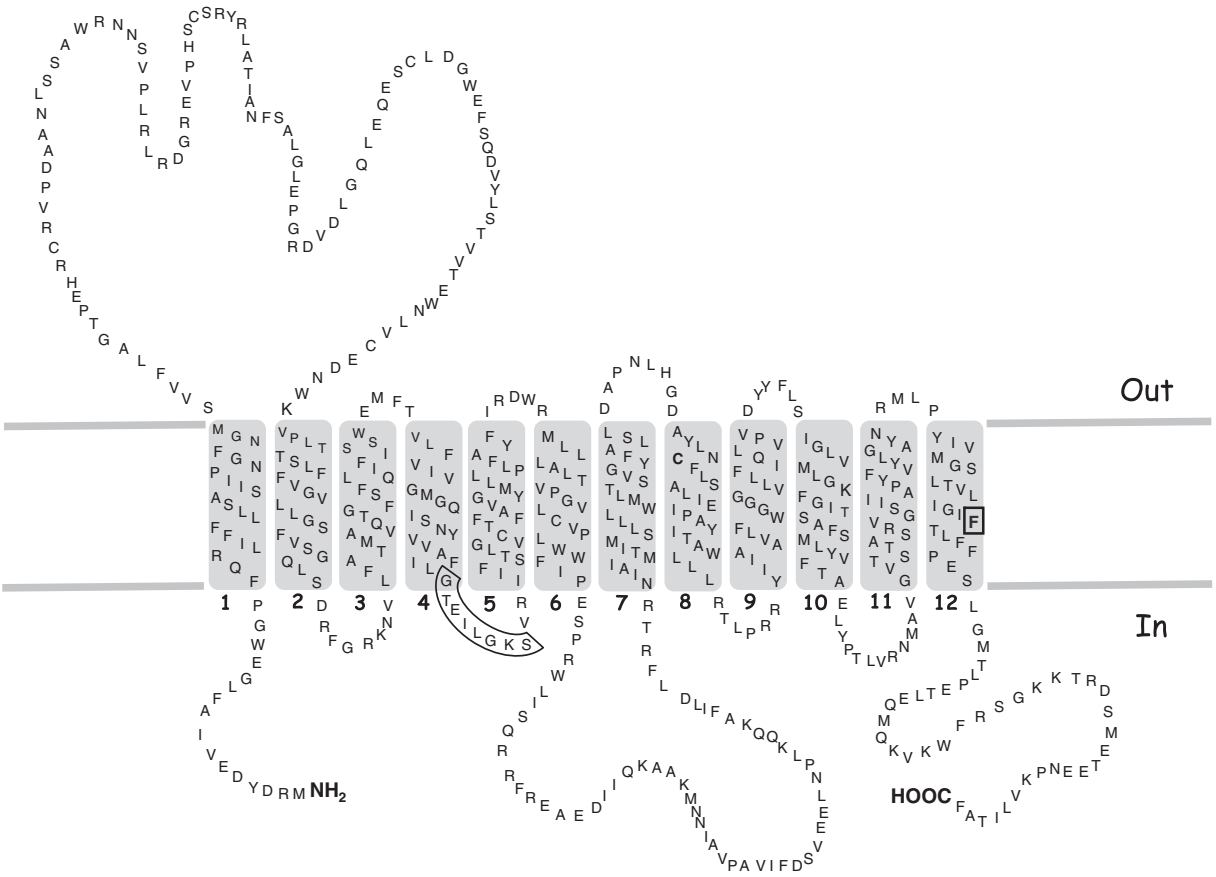


**Fig. 10.** Topology of hOCTN1 with 12 transmembrane α-helices and a large extracellular loop. The model was created on the basis of the TM-PRED prediction software (output format: min 17, max 29) and modified considering the established criteria for trans-membrane proteins. Transmembrane segments are numbered (1–12). Location of the mutated L503F residue of the Crohn's variant OCTN1 is indicated by a box in the transmembrane segment 12. The predicted nucleotide binding motif GTEILGKS is highlighted (boxed).

conformation of the substrate binding site. The physiological implication of the acetylcholine transport function of hOCTN1 can be interpreted in the light of the newly described non-neuronal cholinergic system [9,10]. Indeed, in contrast to the release of neuronal acetylcholine via exocytosis, the mechanisms of acetylcholine trafficking through non-neuronal membranes are still poorly understood. Furthermore VAchT, the vesicular acetylcholine transporter, is not present in all the tissues of the non-neuronal cholinergic system [11]. It has been previously suggested, that OCT1 and OCT2 transporters are involved in acetylcholine transport in placenta [9], in bronchial epithelium and in brain [13]. However, these transporters are not expressed in all the tissues in which acetylcholine is synthesized [9,29,30]. In these tissues, i.e., trachea, kidney, prostate, testis, sperm, spinal cord, bone marrow, muscle cells, granulocytes, lymphocytes and macrophages [13,31] OCTN1 has high level of expression, thus, playing an important role in the acetylcholine trafficking. Other considerations, i.e., the Km's of hOCTN1 and OCT2 [13,30] for acetylcholine are different, hOCTN1 catalyzes efflux, the sub-localizations of OCT2 and OCTN1 are different [14,30], suggest that the concerted action of the different transporters may be essential for the overall regulation of the trafficking of acetylcholine in tissues where both of them are expressed. Furthermore, OCTN1 may be involved in the recycling of acetylcholine when it is not hydrolysed by esterase, especially in non-nervous tissues where acetylcholine esterases have lower activity [29]. Interestingly, it has been recently reported that acetylcholine may play an important role in inducing anti-inflammatory effects via α7-nAChRs which are expressed in several non neuronal tissues [9,29]. To play this autocrine effect, acetylcholine needs to be exported from the cells [29]. The intracellular acetylcholine concentration in non-neuronal tissues has not yet been determined, while in neuronal tissues it has been reported to be around 1 mM [28]. Thus, it is plausible that the autocrine effect may be exerted by the efflux of acetylcholine and, even in the presence of intracellular concentration lower than 1 mM, corresponding about to the Km of the internal side of hOCTN1, the efflux should provide sufficient acetylcholine for high-affinity interaction with the α7-nAChRs receptor. Thus, the efflux function will have importance in this scenario. The inhibition of the transport by polyamine may play an additional role in the inflammatory process. Indeed, spermine concentration increases in tissues during inflammation due to release from dying cells and plays antinflammatory actions [32]. The strong inhibition of hOCTN1 by polyamines may reduce acetylcholine uptake in cells, prolonging its antinflammatory effect concomitantly with that of polyamines. Of note, the export function is specifically impaired in the hOCTN1 mutant 503F which has been correlated with the inflammatory bowel disease and Crohn's disease [14]. The mutation affects the transmembrane segment 12 of the protein (Fig. 10). Similarly, structural alterations of transmembrane segments 4, 7 and 11 of the OCTN2 transporter caused by gene mutations, result in functional impairments [33–35]. In the case of hOCTN1 the substitution of Leu-503 with Phe, reduces the $V_{max}$ without affecting significantly the Km, indicating that the substrate binding site is not modified whereas the conformational changes necessary for substrate efflux from the vesicles are impaired. It is very unlike that the reduced function may be due to reduced incorporation into liposomes, since the uptake catalyzed by the L503F mutant is nearly coincident to that of the wild type (see Fig. 6). The above findings may have broad pathophysiological implications. For instance, given the reported roles of non-neuronal acetylcoline in maintaining epithelial integrity and cell–cell interaction [36], our finding may link defective export of acetylcoline by the hOCTN1 503F variant with a compromised intestinal–pathogen barrier, as it is observed in inflammatory bowel disease. This and similar hypotheses will be object of further experimental

investigation in cellular and animal experimental models. The studies on acetylcholine transport confirmed the previous finding that hOCTN1 may be involved in the transport of γ-butyrobetaine [8] synthesized by the gut microbioma [37,38] in the intestine, where hOCTN1 is highly expressed. It has also been found that acetylcarnitine, but not carnitine, strongly inhibited the transport of acetylcholine confirming that hOCTN1 may also be involved in the transport of acetylated compounds which are important in the metabolic regulation [39].

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## References

[1] I. Tamai, R. Ohashi, J.I. Nezu, Y. Sai, D. Kobayashi, A. Oku, M. Shimane, A. Tsuji, Molecular and functional characterization of organic cation/carnitine transporter family in mice, J. Biol. Chem. 275 (2000) 40064–40072.
[2] S.A. Eraly, J.C. Monte, S.K. Nigam, Novel slc22 transporter homologs in fly, worm, and human clarify the phylogeny of organic anion and cation transporters, Physiol. Genomics 18 (2004) 12–24.
[3] H. Yabuuchi, I. Tamai, J. Nezu, K. Sakamoto, A. Oku, M. Shimane, Y. Sai, A. Tsuji, Novel membrane transporter OCTN1 mediates multispecific, bidirectional, and pH-dependent transport of organic cations, J. Pharmacol. Exp. Ther. 289 (1999) 768–773.
[4] T. Nakamura, K. Yoshida, H. Yabuuchi, T. Maeda, I. Tamai, Functional characterization of ergothioneine transport by rat organic cation/carnitine transporter Octn1 (slc22a4), Biol. Pharm. Bull. 31 (2008) 1580–1584.
[5] D. Grundemann, S. Harlfinger, S. Golz, A. Geerts, A. Lazar, R. Berkels, N. Jung, A. Rubbert, E. Schomig, Discovery of the ergothioneine transporter, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 5256–5261.
[6] A.M. Lamhonwah, I. Tein, Novel localization of OCTN1, an organic cation/carnitine transporter, to mammalian mitochondria, Biochem. Biophys. Res. Commun. 345 (2006) 1315–1325.
[7] M. Galluccio, L. Pochini, L. Amelio, R. Accardi, M. Tommasino, C. Indiveri, Over-expression in *E. coli* and purification of the human OCTN1 transport protein, Protein Expr. Purif. 68 (2009) 215–220.
[8] L. Pochini, M. Scalise, M. Galluccio, L. Amelio, C. Indiveri, Reconstitution in liposomes of the functionally active human OCTN1 (SLC22A4) transporter overexpressed in *Escherichia coli*, Biochem. J. 439 (2011) 227–233.
[9] I. Wessler, C.J. Kirkpatrick, Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans, Br. J. Pharmacol. 154 (2008) 1558–1571.
[10] O.R. Rana, P. Schauerte, R. Kluttig, J.W. Schroder, R.R. Koenen, C. Weber, K.W. Nolte, J. Weis, R. Hoffmann, N. Marx, E. Saygili, Acetylcholine as an age-dependent non-neuronal source in the heart, Auton. Neurosci. 156 (2010) 82–89.
[11] S.M. Parsons, Transport mechanisms in acetylcholine and monoamine storage, FASEB J. 14 (2000) 2423–2434.
[12] K.S. Lips, C. Volk, B.M. Schmitt, U. Pfeil, P. Arndt, D. Miska, L. Ermert, W. Kummer, H. Koepsell, Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium, Am. J. Respir. Cell Mol. Biol. 33 (2005) 79–88.
[13] H. Koepsell, K. Lips, C. Volk, Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications, Pharm. Res. 24 (2007) 1227–1251.
[14] V.D. Peltekova, R.F. Wintle, L.A. Rubin, C.I. Amos, Q. Huang, X. Gu, B. Newman, M. Van Oene, D. Cescon, G. Greenberg, A.M. Griffiths, P.H. St George-Hyslop, K.A. Siminovitch, Functional variants of OCTN cation transporter genes are associated with Crohn disease, Nat. Genet. 36 (2004) 471–475.
[15] R. Kramer, C. Heberger, Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column, Biochim. Biophys. Acta 863 (1986) 289–296.
[16] F. Palmieri, M. Klingenberg, Direct methods for measuring metabolite transport and distribution in mitochondria, Methods Enzymol. 56 (1979) 279–301.
[17] C. Brizio, R. Brandsch, D. Bufano, L. Pochini, C. Indiveri, M. Barile, Over-expression in *Escherichia coli*, functional characterization and refolding of rat dimethylglycine dehydrogenase, Protein Expr. Purif. 37 (2004) 434–442.
[18] I. Tamai, H. Yabuuchi, J. Nezu, Y. Sai, A. Oku, M. Shimane, A. Tsuji, Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1, FEBS Lett. 419 (1997) 107–111.
[19] L. Pochini, F. Oppedisano, C. Indiveri, Reconstitution into liposomes and functional characterization of the carnitine transporter from renal cell plasma membrane, Biochim. Biophys. Acta 1661 (2004) 78–86.
[20] F. Oppedisano, L. Pochini, M. Galluccio, M. Cavarelli, C. Indiveri, Reconstitution into liposomes of the glutamine/amino acid transporter from renal cell plasma membrane: functional characterization, kinetics and activation by nucleotides, Biochim. Biophys. Acta 1667 (2004) 122–131.
[21] F. Oppedisano, C. Indiveri, Reconstitution into liposomes of the B ° -like glutamine-neutral amino acid transporter from renal cell plasma membrane, Biochim. Biophys. Acta 1778 (2008) 2258–2265.
[22] F. Palmieri, C. Indiveri, F. Bisaccia, V. Iacobazzi, Mitochondrial metabolite carrier proteins: purification, reconstitution, and transport studies, Methods Enzymol. 260 (1995) 349–369.
[23] C. Indiveri, Studying amino acid transport using liposomes, Meth. Mol. Biol. 606 (2010) 55–68.
[24] M. Quick, E.M. Wright, Employing *Escherichia coli* to functionally express, purify, and characterize a human transporter, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 8597–8601.
[25] T. Keller, D. Schwarz, F. Bernhard, V. Dotsch, C. Hunte, V. Gorboulev, H. Koepsell, Cell free expression and functional reconstitution of eukaryotic drug transporters, Biochemistry 47 (2008) 4552–4564.
[26] S. Leviatan, K. Sawada, Y. Moriyama, N. Nelson, Combinatorial method for overexpression of membrane proteins in *Escherichia coli*, J. Biol. Chem. 285 (2010) 23548–23556.
[27] T. Komatsu, M. Hiasa, T. Miyaji, T. Kanamoto, T. Matsumoto, M. Otsuka, Y. Moriyama, H. Omote, Characterization of the human MATE2 proton-coupled polyspecific organic cation exporter, Int. J. Biochem. Cell Biol. 43 (2011) 913–918.
[28] A.M. Ojeda, N.G. Kolmakova, S.M. Parsons, Acetylcholine binding site in the vesicular acetylcholine transporter, Biochemistry 43 (2004) 11163–11174.
[29] I. Wessler, H. Kilbinger, F. Bittinger, R. Unger, C.J. Kirkpatrick, The non-neuronal cholinergic system in humans: expression, function and pathophysiology, Life Sci. 72 (2003) 2055–2061.
[30] G. Ciarimboli, Role of organic cation transporters in drug-induced toxicity, Expert Opin. Drug Metab. Toxicol. 7 (2011) 159–174.
[31] K. Kawashima, T. Fujii, Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance, J. Pharmacol. Sci. 106 (2008) 167–173.
[32] G. Bjelakovic, I. Stojanovic, T. Jevtovic Stoimenov, D. Pavlovic, G. Kocic, S. Rossi, C. Tabolacci, J. Nikolic, D. Sokolovic, L. Bjelakovic, Metabolic correlations of glucocorticoids and polyamines in inflammation and apoptosis, Amino Acids 39 (2010) 29–43.
[33] R. Rodrigues Pereira, H.R. Scholte, I.E. Luyt-Houwen, M.H. Vaandrager-Verduin, Cardiomyopathy associated with carnitine loss in kidneys and small intestine, Eur. J. Pediatr. 148 (1988) 193–197.
[34] P. Seth, X. Wu, W. Huang, F.H. Leibach, V. Ganapathy, Mutations in novel organic cation transporter (OCTN2), an organic cation/carnitine transporter, with differential effects on the organic cation transport function and the carnitine transport function, J. Biol. Chem. 274 (1999) 33388–33392.
[35] Y. Wang, F. Taroni, B. Garavaglia, N. Longo, Functional analysis of mutations in the OCTN2 transporter causing primary carnitine deficiency: lack of genotype–phenotype correlation, Hum. Mutat. 16 (2000) 401–407.
[36] S.A. Grando, Biological functions of keratinocyte cholinergic receptors, J. Investig. Dermatol. Symp. Proc. 2 (1997) 41–48.
[37] C.J. Rebouche, H. Seim, Carnitine metabolism and its regulation in microorganisms and mammals, Annu. Rev. Nutr. 18 (1998) 39–61.
[38] P. Stenmark, D. Gurmu, P. Nordlund, Crystal structure of CaiB, a type-III CoA transferase in carnitine metabolism, Biochemistry 43 (2004) 13996–14003.
[39] K.L. Guan, Y. Xiong, Regulation of intermediary metabolism by protein acetylation, Trends Biochem. Sci. 36 (2011) 108–116.