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Lorena Pochini, Mariafrancesca Scalise, Michele Galluccio, Linda Amelio, Cesare Indiveri. Reconstitution in liposomes of the functionally active human OCTN1 (SLC22A4) transporter over-expressed in *E. coli*. *Biochemical Journal*, 2011, 439 (2), pp.227-233. 10.1042/BJ20110544 . hal-00628665

**HAL Id: hal-00628665**

**<https://hal.science/hal-00628665>**

Submitted on 4 Oct 2011

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**Reconstitution in liposomes of the functionally active human OCTN1 (SLC22A4) transporter over-expressed in E. coli.**

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Key words: OCTN1; transport; reconstitution; tetraethylammonium

Abbreviations used: TEA, tetraethylammonium; PLP, pyridoxal 5-phosphate; ANTP, adenosine 5'-( $\beta,\gamma$ -imido)triphosphate; GABA,  $\gamma$ -Aminobutyric acid; MTSES, sodium (2-sulphonatoethyl)methanethiosulfonate; p-OHMB, p-hydroxymercuribenzoate; NEM, N-ethylmaleimide.

## SYNOPSIS

The hOCTN1 transporter over-expressed in *E. coli* and purified by Ni-chelating chromatography has been reconstituted in liposomes by detergent removal with a batch-wise procedure. The reconstitution was optimised with respect to the protein concentration, the detergent/phospholipid ratio and the time of incubation with Amberlite XAD-4 resin. Time dependent [<sup>14</sup>C]tetraethylammonium, [<sup>3</sup>H]carnitine or [<sup>3</sup>H]ergothioneine uptake was measured in proteoliposomes with activities ratio of 8.0 : 1.3 : 1.0, respectively. Optimal activity was found at pH 8.0. The transport depended on intraliposomal ATP. [<sup>14</sup>C]tetraethylammonium transport was inhibited by several compounds; the most effective were acetylcholine and  $\gamma$ -butyrobetaine, followed by acetylcarnitine and tetramethylammonium. Reagents such as pyridoxal 5-phosphate, MTSES and mercurials strongly inhibited the transport. From kinetic analysis of tetraethylammonium transport a  $K_m$  of 0.77 mM was calculated. Acetylcholine and  $\gamma$ -butyrobetaine behaved as competitive inhibitors of tetraethylammonium transport with  $K_i$  values of 0.44 and 0.63 mM, respectively.

## INTRODUCTION

Membrane proteins represent more than 20 % of proteomes of all organisms [1]. Transporters, which belong to this class of proteins, are responsible of nutrient uptake and trafficking, and elimination of catabolites. Several transport systems are essential for life as demonstrated by the occurrence of human pathologies caused by defects of their function [2-7]. In spite of the importance of the transport systems in metabolism of higher organisms including humans, the study of these proteins has a large delay respect to the study of soluble enzymes. This is mainly due to the hydrophobic properties of transport proteins which make them difficult to handle and to the vectorial type of the catalyzed reactions, which require the insertion of the proteins into a membrane system to study its function. So far, the functional data on animal and human transport proteins derives mostly from expression in minute amount of the protein in oocytes or other intact cell systems in which the transport activity is followed in comparison with controls. A different strategy for studying animal transport proteins consists in the heterologous expression of the proteins followed by insertion in appropriate model membranes to assay the transport function. Heterologous expression allows also to obtain much larger amount of proteins, which can be used for structural studies. However, this strategy presents several challenges: difficulties of over-expression of larger hydrophobic proteins (more than 300 amino acids); propensity of the proteins to aggregate; difficulties in refolding and/or reconstitution in model membranes. Recently, the over-expression in *E. coli* and large scale purification of the human OCTN1 transporter was obtained [8]. This transporter belongs to a small subfamily of proteins which share more than 66 % identity with each other [8]. The members, OCTN1, OCTN2 and OCTN3 have been identified only in vertebrates [9]. While OCTN1 and OCTN2 genes have been identified in *H. sapiens*, OCTN3 gene has been identified only in mouse. The function of the proteins coded by the OCTN genes is very probably related with carnitine homeostasis. Indeed alterations of the OCTN2 gene lead to primary carnitine deficiency in humans [10]. The OCTN2 protein is the most widely characterized at the functional level among the members of the subfamily. Studies performed in brush-border vesicles [11], in intact cell systems expressing the transport protein [12, 13] and in proteoliposomes reconstituted with the protein extracted from rat kidney [14], clarified that OCTN2 transports carnitine in a variety of tissues and organisms. The OCTN1 protein has been studied in cell systems such as oocytes or HEK293 cells, expressing the transporter. In these studies it was described that OCTN1 catalyzed mainly transport of TEA [12, 15-18]. More recently it was reported that the main substrate of OCTN1 is ergothioneine, not TEA [19]. Other authors showed that OCTN1 transports carnitine and is localized in the inner mitochondrial membrane [20]. Thus, some properties concerning the function and the biogenesis of this transporter are still controversial. In this paper we describe a procedure for the reconstitution of the hOCTN1 over-expressed in *E. coli* and the functional characterization of the human protein in

terms of transported substrates and specificity towards inhibitors and regulators. The experimental data confirms most of the data described in intact cell systems and, more importantly, reveal novel properties of the transporter which shed new light in the metabolic function of this protein.

## EXPERIMENTAL

### Materials

Amberlite XAD-4, egg yolk phospholipids (3-sn-phosphatidylcholine from egg yolk) and Triton-X100 were purchased from Fluka; Tetraethylammonium [ethyl-1-<sup>14</sup>C] chloride, Carnitine, L-[N-methyl-<sup>3</sup>H] hydrochloride, and Ergothioneine-[<sup>3</sup>H] were from Hartmann analytic; Sephadex G-75, L-carnitine, tetraethylammonium chloride from Sigma, ergothioneine from Santa Cruz Biotechnology. All the other reagents were of analytical grade.

### Reconstitution of the hOCTN1 transporter into liposomes.

hOCTN1 was over-expressed in *E.coli* and purified as previously described [8]. The purified hOCTN1 was reconstituted by removing the detergent from mixed micelles containing detergent, protein and phospholipids by incubation with Amberlite XAD-4 in a batch-wise procedure [21-22]. The composition of the initial mixture used for reconstitution (except when differently indicated) was: 180  $\mu$ l of the purified protein (6  $\mu$ g protein in 0.1% Triton-X100), 120  $\mu$ l of 10 % Triton X-100, 120  $\mu$ l of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as previously described [23], 16 mM ATP, 10 mM Tris/HCl (pH 8.0) in a final volume of 700  $\mu$ l. After vortexing, this mixture was incubated with 0.55 g Amberlite XAD-4 under rotatory stirring (1400 rpm) at room temperature for 45 min.

### Transport measurements.

550  $\mu$ l of proteoliposomes were passed through a Sephadex G-75 column (0.7 cm diameter x 15 cm height) preequilibrated with 5 mM Tris/HCl (pH 8.0). 550  $\mu$ l of proteoliposomes were collected from these columns and divided in aliquots (samples) of 100  $\mu$ l. Transport was started by adding the indicated concentrations of [<sup>14</sup>C]Tetraethylammonium (TEA) or other radioactive substrates to the proteoliposomes 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 [24]. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100  $\mu$ l) was passed through a Sephadex G-75 column (0.6 cm diameter x 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 less than 15 % with respect to the PLP sensitive TEA transport. The initial rate of transport expressed as nmol/min/mg protein, was measured by stopping the reaction after 5 min, i.e., within the initial linear range of [<sup>14</sup>C]TEA uptake into the proteoliposomes (see Fig. 4). Kinetic constants values were determined using the Grafit (version 5.0.3) software.

### 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 [25]. The internal volume of the proteoliposomes was measured as previously described [26].

## RESULTS

### Optimal conditions for reconstitution

The hOCTN1 after purification was solubilized in 0.1 % Triton X-100 [8]. A first attempt of reconstitution was performed by the cyclic detergent removal procedure used for other plasma membrane transporters [14, 27, 28]. This procedure is based on the removal of detergent from mixed micelles of phospholipid, detergent and protein by repeated chromatography on the

hydrophobic resin Amberlite XAD-4 which specifically bind non ionic detergents. Transport was measured as [ $^{14}\text{C}$ ]TEA uptake in the formed proteoliposomes. In this experimental condition the transport activity of hOCTN1 was very low (not shown). In an attempt to improve the transport activity of the protein the procedure of detergent removal was changed, using a batch-wise method [20-22] instead of the chromatography. Indeed, this method led to higher and reproducible transport. Thus, the procedure was further optimised by adjusting the parameters that influence the efficiency of transport protein incorporation into the liposomes [14, 27, 28]. In these experiments both the transport activity and the internal volume of the proteoliposomes were measured. The first parameter gives information on the specific activity of the transporter, whereas the second is correlated to the efficiency of proteoliposome formation.

The influence of the protein concentration on the reconstitution efficiency was studied. It was found that the transport activity increased almost linearly with the protein concentration up to 8-10  $\mu\text{g/ml}$ . Above these values, a reduction of the transport activity was measured. The intraliposomal volume was nearly not influenced by the protein concentration, indicating that the protein did not interfere with the formation of liposomes. Thus, for the experiments, we have chosen a protein concentration of 8  $\mu\text{g/ml}$ . The dependence of transport on the detergent/lipid ratio showed that optimal activity was obtained in the range 0.9-1.3 detergent/lipid. The internal volume slightly decreased with increasing detergent/lipid ratios. The dependence of transport on the time of incubation with the Amberlite XAD-4 resin was further measured. 45 min incubation represented the optimal condition. The internal volume decreased by further increasing the time of incubation (for further details see the figures in the supplementary section).

#### Functional characterization

The uptake of [ $^{14}\text{C}$ ]TEA in liposomes reconstituted with hOCTN1 was measured as function of the time (Fig. 1). As control, the [ $^{14}\text{C}$ ]TEA associated with liposomes without incorporated protein was also measured. [ $^{14}\text{C}$ ]TEA uptake in proteoliposomes was much higher than in the control (liposomes without protein; not shown). The specific transport activity of hOCTN1 referred to the mg proteins, was calculated from the difference between the uptake in the presence and absence of protein. Since PLP was found to strongly inhibit the transporter (see below), this compound was tested as stop inhibitor (see Materials and methods section). The time course of the specific transport activity calculated from the experimental data subtracted by the samples inhibited at time zero, was nearly coincident with that of the specific transport activity calculated by subtracting the controls without protein. This indicated that the stop inhibitor procedure could be applied to measure the specific transport. In the same experiment the uptake of [ $^{14}\text{C}$ ]TEA in proteoliposomes containing internal TEA was also measured to gain insights in the transport mode, i.e., whether hOCTN1 catalysed uniport or antiport of TEA. As shown by the figure, the specific transport in the presence of intraliposomal TEA was nearly coincident with that in the absence of internal substrate, indicating that the transport system functions according to a uniport mode. The addition of NaCl or Na acetate from 5 to 100 mM to the proteoliposomes together with the labelled substrate did not stimulate the uptake (not shown) indicating that the transport of TEA was sodium independent. Transport was also measured in proteoliposomes reconstituted with boiled protein. In this case, the specific transport was much lower than that measured with native protein, indicating that the transport was protein-mediated. The data of the time course of TEA uptake fitted a first order rate equation. The initial rate of the transport process derived from the equation as the product of  $k$  (the first order rate constant) and the transport at the equilibrium was 2.6 nmol/min/mg protein. This value was very similar to that obtained as described in Materials and Methods as an approximation to the initial rate, using the first experimental points.

It was previously reported that the mouse or human OCTN1 transport system catalyses in cells, transport of TEA, carnitine [12,16,20] and/or ergothioneine [19]. Thus the ability of the reconstituted hOCTN1 to mediate transport of these compounds have been also tested using [ $^3\text{H}$ ]carnitine and [ $^3\text{H}$ ]ergothioneine. The results of the time dependence of the uptake of these compounds in proteoliposomes are depicted in Fig. 2. Uptake of both carnitine and ergothioneine



increased with the time. The equilibrium was reached at similar values for both substrates. As found in the case of TEA, PLP strongly inhibited the transport of both [<sup>3</sup>H]carnitine and [<sup>3</sup>H]ergothioneine. Also in these cases, the data fitted in a first order rate equation. The lower correlation of the experimental data with the first order process respect to the TEA uptake, was due to the higher fluctuations (SD values) of the measurements caused by the lower transport activity. Indeed, the values of initial rate derived from the equation were 0.33 and 0.43 nmol/min/mg protein for carnitine and ergothioneine, respectively, i.e., 8 and 6 times lower than the transport rate calculated for TEA. The data indicated that both carnitine and ergothioneine were transported with lower efficiency than TEA. The dependence of the transport of [<sup>14</sup>C]TEA and [<sup>3</sup>H]carnitine in the proteoliposomes on the pH was studied. As shown in Fig. 3, the transport of TEA strongly increased by increasing the pH from 6.0 to 7.5, then slightly increased at pH 8.0 and remained nearly constant up to pH 8.5. A similar dependence was observed for carnitine (Fig. 3). At more alkaline pH the unspecific permeability (uptake without protein or with stop inhibitor) of proteoliposomes to TEA and carnitine largely increased; thus, the net transport could not be accurately determined. The dependence of transport function on intraliposomal ATP was studied. For this purpose separate pools or proteoliposomes were reconstituted with internal ATP at concentrations ranging from 0 to 16 mM. As shown by Fig. 4, the transport activity increased with increasing ATP concentration. The experimental data showed a saturation behaviour up to 8 mM ATP. However, by further increasing the ATP concentration the transport activity still increased reaching a value of 2 nmol/min/mg protein at 16 mM ATP. A similar effect was exerted by the non hydrolysable analogue ANTP, indicating that the effect of activation was not due to ATP hydrolysis. AMP and PPi exerted a lower activation effect of about 60 % respect to that exerted by ATP, whereas adenosine did not exert significant effect (Fig. 5). UTP and GTP activated the transporter as ATP, indicating that the site on the hOCTN1 protein was not specific for the base moiety. No variations were observed by the addition of Mg<sup>++</sup> together with internal nucleotides and no effects were exerted by nucleotides on the external side of the proteoliposomes (not shown).

The specificity of the transporter towards potential substrates and/or inhibitors has been tested. Among potential substrates several molecules which share the presence of a cation moiety in the molecule have been tested. As shown in table 1, among the molecules tested as substrates, carnitine, betaine, ergothioneine, creatinine, creatine and GABA did not exerted any effect on the transport of TEA at concentrations up to 1 mM. These data indicated that these molecules did not compete with TEA for transport. The inhibition by higher carnitine concentrations was also tested: 33 % inhibition was observed with 15 mM carnitine. Whereas, choline, acetylcholine, acetylcarnitine,  $\gamma$ -butyrobetaine and tetramethylammonium exerted significant inhibition, i.e., should be transported. Reagents which react with specific amino acid residues of proteins have been tested as inhibitors. As shown in Table 2, hOCTN1 was strongly inhibited by HgCl<sub>2</sub> and, at a lower extent, by mersalyl, p-OHMB and MTSES, which are specific for SH residues. PLP, which is specific for NH<sub>2</sub> residues, strongly inhibited the transporter. On the contrary the SH reagent NEM and the His reagent diethylpirocarbonate did not inhibit transport. In another experiment the inhibition of [<sup>3</sup>H]carnitine transport by TEA was measured. 50% inhibition was observed at 2 mM TEA; complete inhibition of the carnitine transport was observed at 20 mM TEA.

### Kinetics of TEA transport

To obtain kinetic data of the hOCTN1 transporter, the dependence of the transport rate on the external substrate concentration was studied by changing the concentration of [<sup>14</sup>C]TEA added to the proteoliposomes. The experimental data represented in a double reciprocal plot were interpolated by a straight line (Fig. 6). The half saturation constant derived from the equation was  $0.77 \pm 0.2$  mM. The V<sub>max</sub> was  $16.1 \pm 3.9$  nmol/min/mg protein (from 3 different experiments). To further investigate the interaction of acetylcholine,  $\gamma$ -butyrobetaine and carnitine with the transporter, inhibition kinetic studies were performed. In these experiments the transport rate of [<sup>14</sup>C]TEA was studied as dependence of the TEA concentrations in the presence of the various

compounds. As shown by Fig. 9, both acetylcholine (Fig. 6 A) and  $\gamma$ -butyrobetaine (Fig. 6 B) inhibited the transport according with a competitive mode with respect to TEA. The  $K_i$  were calculated from the interpolation of the data: their values were  $0.44 \pm 0.15$  and  $0.63 \pm 0.06$  mM for acetylcholine and  $\gamma$ -butyrobetaine, respectively. Whereas, in the case of carnitine (Fig. 6 C), a non competitive behaviour was observed with a much higher calculated  $K_i$ ,  $49 \pm 11$  mM.

## DISCUSSION

While several mitochondrial transporters over-expressed in bacteria have been reconstituted in liposomes [29], very few examples of reconstitution of mammalian plasma membrane transporters expressed in bacteria, are available [30-32]. This is due to both difficulties in over-expressing and refolding plasma membrane transporters which are larger than mitochondrial ones. Indeed, the experimental model of reconstitution in liposomes is the most up-to-date for obtaining reliable information on the function of transporters. This system allows to assay the activity of purified transporters in absence of interferences caused, in intact cells, by other transport systems which are present in the same membrane and by intracellular enzymes which may chemically modify the substrates taken up [14, 27,28]. Furthermore, the reconstitution is an essential tool for studying heterologously expressed human transporters which cannot be studied after extraction from tissues as in the case of animal transporters [14,27,28]. The hOCTN1 transporter over-expressed in *E. coli* and purified in a large scale [8] has been reconstituted in liposomes and functionally characterized. The data on the specificity for substrates of reconstituted hOCTN1 are in agreement with previous findings [12, 16-18], i.e., the transport activity of TEA is higher than that of carnitine and ergothioneine. Also the pH dependence of transport in proteoliposomes was very similar to that previously described in cells [15]. All together, these findings are somewhat in contrast with other reports in which the transport of ergothioneine mediated by OCTN1 was found to be much higher than TEA and carnitine and optimal activity was found at acidic pH [19]. The discrepancy in the case of cells may be due, as previously suggested [18], to different experimental conditions in the measurement of transport. In the case of proteoliposomes, the discrepancy may be due to absence of interferences by other molecular systems and/or to the lack of regulatory factors, which may exert effects in the cell. Indeed, the reconstituted protein is in a purified state. Thus, in agreement with several previous studies [12, 16-18] we can hypothesize that under the basic state, hOCTN1 catalyzes mainly organic cation transport. Other experimental data obtained by means of the proteoliposome system, demonstrated that acetylcarnitine, acetylcholine, choline and  $\gamma$ -butyrobetaine inhibits the transport of TEA catalysed by hOCTN1. Thus, these compounds might be transported by hOCTN1, being possible physiological substrates. As further support to this hypothesis it has been found that acetylcholine, and  $\gamma$ -butyrobetaine are competitive inhibitors of TEA transport. The specificity for acetylcholine suggests an involvement of the transporter in peripheral nervous tissue function. Indeed, hOCTN1 has been found to be more expressed in peripheral nervous tissue than in brain [15]. Interestingly, synthesis of acetylcholine has been demonstrated also in kidney, placenta, skin, bladder [33], heart [34]. hOCTN1 is also highly expressed in the intestine where it may play a role in the transport of compounds produced by the gut microbioma. Indeed, some enterobacteria excrete  $\gamma$ -butyrobetaine [35, 36] which can be used for carnitine synthesis in humans [10]. These functions which are typical of higher animals, correlates well with the finding that SLC22 family, including OCTNs, emerged after the divergence between invertebrates and vertebrates [9]. The effects caused by mercuric compounds, other SH reagents and by PLP suggested that some Cys and Lys residues of the protein must be involved in the transport function. Moreover, we have demonstrated that ATP present in the internal proteoliposomal compartment activates the transporter. The effect is not due to phosphorylation of the protein or primary active transport activated by ATP hydrolysis, since the non hydrolysable analogous shows the same effect of ATP. In agreement, it was previously described in cell systems, that hOCTN1 showed a reduced transport function in cell depleted of ATP [15]. Other triphosphate nucleosides exerted the same effect. Similar unspecific regulation

by phosphate nucleosides was previously observed for other transporters like the mitochondrial UCP [40] and the glutamine/neutral amino acid transporter ASCT2 [27]. The activation effect of ATP showed a biphasic behaviour. The first optimum of activity was observed at concentrations corresponding to the physiological level of ATP [41]. However, a further stimulatory effect was observed at higher concentrations. This may be interpreted in the light of the finding of microdomain regions close to the membrane, in which ATP concentration is in the order of several millimolar, due to the presence of peripheral mitochondria [39, 42-43]. On the basis of the sidedness of ATP regulation it can be deduced that the transporter is inserted into the proteoliposomal membrane with the same orientation of the cell membrane as previously found for other reconstituted plasma membrane transport systems [14, 27, 28]. The mechanism of activation by ATP will be investigated in further studies to clarify whether it influences the  $V_{max}$  or  $K_m$  or both of the transporter. The weak inhibition of TEA transport by carnitine and vice versa of carnitine transport by TEA, together with the finding that carnitine inhibits TEA transport non competitively and it is transported by OCTN1 may be interpreted with the existence of two independent binding sites for TEA and carnitine or different points of recognition (subsites) within a larger pocket. Similar models had been previously proposed for other transporters. As examples, two independent binding sites were proposed for the mitochondrial dicarboxylate carrier [44] or different points of recognition for different substrates were proposed for the OCTN2 transporter [45].

## FUNDING

This work was supported by grants from Ministero dell'Università e della Ricerca (PRIN and FIRB) and from University of Calabria (ex 60 % 2009-2010).

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### Legends to figures

Fig. 1. Time course of TEA uptake by reconstituted proteoliposomes. The reconstitution was performed as described in Materials and methods. Transport was started adding 0.1 mM [ $^{14}\text{C}$ ]TEA at time zero to proteoliposomes ( $\bullet$ ,  $\blacktriangledown$ ,  $\blacktriangle$ ) or proteoliposomes reconstituted with protein treated for 20 min at 100 °C ( $\blacksquare$ ). In ( $\blacktriangle$ ) 2 mM TEA was present inside the proteoliposomes. In ( $\blacktriangledown$ ) the transport reaction was stopped at the indicated times and the specific transport activity (referred to the mg proteins) was calculated according to the inhibitor stop-method as described in Materials and methods. In ( $\bullet$ ,  $\blacktriangle$ ,  $\blacksquare$ ) the transport reaction was stopped 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 protein. The values are means  $\pm$  S.D. from three experiments.

Fig. 2. Time course of carnitine and ergothioneine uptake by reconstituted proteoliposomes. The reconstitution was performed as described in Materials and methods. Transport was started adding 0.1 mM [ $^3\text{H}$ ]carnitine ( $\circ$ ) or [ $^3\text{H}$ ]ergothioneine ( $\bullet$ ) to the proteoliposomes at time zero. 2 mM PLP was added to the proteoliposomes at time zero together with [ $^3\text{H}$ ]carnitine ( $\square$ ) or [ $^3\text{H}$ ]ergothioneine ( $\blacksquare$ ). The values are means  $\pm$  S.D. from three experiments.

Fig. 3 Effect of pH on the reconstituted hOCTN1. The reconstitution was performed as described in Materials and methods except that 10 mM Tris/HCl at the indicated pH was used. Transport was started adding 0.1 mM [ $^{14}\text{C}$ ]TEA ( $\circ$ ; left y-axis) or [ $^3\text{H}$ ]carnitine ( $\bullet$ ; right y-axis) in 5 mM Tris/HCl buffer at the indicated pH to proteoliposomes. The values are means  $\pm$  S.D. from three experiments.

Fig. 4 Dependence of TEA transport on the concentration of intraliposomal ATP. The reconstitution was performed as described in Materials and methods except that the indicated concentrations of ATP were added to the reconstitution mixture. Transport was started adding 0.1mM [ $^{14}\text{C}$ ]TEA to proteoliposomes. The values are means  $\pm$  S.D. from three experiments.

Fig. 5. Effect of different nucleotides on hOCTN1. The reconstitution was performed as described in Materials and methods except that 5 mM of ATP or AMP or PPi or adenosine or UTP or GTP were present in the intraliposomal compartment. Transport was started adding 0.1 mM [ $^{14}\text{C}$ ]TEA to proteoliposomes. The values are means  $\pm$  S.D. from three experiments.

Fig. 6. Kinetic analysis of the inhibition of the reconstituted transporter by acetylcholine and  $\gamma$ -butyrobetaine. The transport rate was measured, as described in Materials and methods adding [ $^{14}\text{C}$ ]TEA at the indicated concentrations to proteoliposomes in the absence ( $\circ$ ) or presence ( $\bullet$ ) of 0.8 mM acetylcholine (A),  $\gamma$ -butyrobetaine (B) or 15 mM carnitine (C). Data were plotted according to Lineweaver-Burk as reciprocal transport rate vs reciprocal TEA concentration. The values are means  $\pm$  S.D. from three experiments.



















