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Na^+-dependent and Na^+-independent betaine transport across the apical membrane of rat renal epithelium

Mercedes Cano, María L. Calonge *, Anunciación A. Ilundáin

Department of Physiology, University of Seville, Spain

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ABSTRACT

The low renal excretion of betaine indicates that the kidney efficiently reabsorbs the betaine filtered by the glomeruli but the mechanisms involved in such a process have been scarcely investigated. We have detected concentrative and non-concentrative betaine transport activity in brush-border membrane vesicles (BBMV) from rat renal cortex and medulla. The concentrative system is the Sodium/Imino-acid Transporter 1 (SIT1) because it is Na^+- and Cl^--dependent, electrogenic and is inhibited by an anti-SIT1 antibody. Its apparent affinity constant for betaine, K_t, is 1.1 ± 0.5 mM and its maximal transport velocity, V_{max}, 0.5 ± 0.1 nmol betaine/mg protein/s. Inhibitors of the Na^+/Cl^-/betaine uptake are L-proline (75%) and cold betaine, L-carnitine and choline (40–60%). Neither creatine, TEA, taurine, β-alanine, GABA nor glycine significantly inhibited Na^+/Cl^-/betaine uptake. The non-concentrative betaine transport system is Na^+- and H^+-independent, electroneutral, with a K_t for betaine of 47 ± 7 μM and a V_{max} of 7.8 ± 1 pmol betaine/mg protein/s. Its transport activity is nearly abolished by betaine, followed by L-carnitine (70–80%) and proline (40–50%), but a difference from the Na^+/Cl^-/betaine transport is that it is inhibited by TEA (approx. 50%) and unaffected by choline. The underlying carrier functions as an antiporter linking betaine entry into the BBMV with the efflux of either L-carnitine or betaine, an exchange unaffected by the anti-SIT1 antibody. As far as we know this is the first work reporting that betaine crosses the apical membrane of rat renal epithelium by SIT1 and by a Na^+- and H^+-independent transport system.

1. Introduction

The quaternary ammonium compound betaine [2(N,N,N-trimethyl) ammonium acetate] is a human nutrient with known physiological functions [see 1 for review]. It is utilized as an osmolyte that under osmotic stress is accumulated within the cells to molar concentrations. Cells of the renal medulla are exposed to high concentrations of NaCl and urea during antidiuresis and they adapt to external hyperosmotic stress by accumulating low monovalent inorganic ions and organic osmolytes such as betaine. The betaine accumulated by the renal medulla mainly comes from exogenous origin and enters the epithelial cells across their basolateral membrane via the Na^+ and Cl^--dependent betaine transporter (BGT1), that transports γ-aminobutyric acid (GABA) with higher affinity than betaine [2,3]. Betaine also functions as a source of methyl groups, gaining further importance as methyl donor in pathologic situations associated with hyperhomocysteinaemia [see 1 for review]. Involvement of betaine in reducing inflammation, in epigenetics and in athletic performance has been also suggested [1].

Betaine is obtained from the diet by rapid intestinal absorption, mainly by the duodenum [4], and the following two observations indicate that the kidney plays a key role in maintaining betaine serum levels: i) the fractional excretion of betaine is low (<6%) [5,6], even at high betaine serum levels [7], and ii) renal diseases elevate betaine excretion and decrease its serum concentration [6,8]. These findings also reveal that the betaine filtered in the glomeruli is efficiently reabsorbed along the nephron, but the mechanism(s) involved in such process has been scarcely investigated and the routes proposed depend on either the nephron tubule or the species. Pummer et al. [9] found, in rat descending limbs of short Henle's loops, an apical, proline-preferring carrier that transports betaine in a diuresis-modulated manner. Studies using brush-border membrane vesicles (BBMV) from rabbit proximal tubule revealed the presence of two non-betaine specific, electrogenic, betaine transporters that were either Na^+- or H^+-sensitive [10], with apparent affinity constants for betaine, K_t, of 4.1 mM and 2.8 mM, respectively. The transport of betaine by either system was inhibited by proline, whereas glycine blocked the H^+-sensitive betaine transport activity and had little effect on the Na^+-sensitive component. Later studies performed in other cell types showed that the Na^+-independent,

Abbreviations: BBMV, brush-border membrane vesicles; Kt, apparent affinity constant; Vmax, maximal transport velocity; SIT1, Sodium/Imino-acid Transporter 1; TEA, tetraethylammonium; GABA, gamma-aminobutyric acid; BGT1, betaine/GABA transporter 1; PAT, proton-coupled amino acid transporter; OCTN, organic cation/carnitine transporter; DMSO, dimethyl sulfoxide; OAT, organic anion transporter; APC, amino acid/polyamine/organocation; CaiT, carnitine transporter; TALH, thick ascending limb of Henle's loop

* Corresponding author at: Dpto. Fisiología, Universidad de Sevilla, C/Profesor García González, n° 2, 41012 Sevilla, Spain.
E-mail address: calonge@us.es (M.L. Calonge).

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pH-dependent transport system, currently known as PAT1 (proton-coupled amino acid transporter 1), is a low-affinity transporter (K_t = 1–10 mM) of proline, hydroxyproline and betaine, among others [see 11 for a review].

Although the Cl^- requirement of the apical Na^+-dependent betaine transport activity has not been detected in kidney [10], this transport activity could be mediated by the IMINO transport system that transports proline across the apical membrane of rat proximal tubule in a Na^+- and Cl^--dependent manner [12]. This transporter has been cloned from rat, human and mouse [13,14]. The human and rat SLC6a20 gene encodes SIT1 (Sodium/Imino-acid Transporter), a member of the SLC6 Na^+- and Cl^--dependent amino acid and neurotransmitter transporter family [14]. Either partial [13] or total [14] Cl^--dependency of SIT1-mediated proline and betaine transport has been measured in oocytes. The mouse has two homologues named XT3 and XT3s1 [13]. XT3s1 mRNA has been detected in the brain, kidney, small intestine, thymus, spleen and lung [13] and the protein at the apical membrane of mice proximal tubule [15], whereas XT3 mRNA prevails in the kidney and lung [13]. In oocytes XT3s1 expression, but not that of XT3, induces a Na^+- and Cl^--dependent amino and imino acid transport activity [14].

While studying L-carnitine transport across the apical membrane of rat renal epithelium we found that betaine is transported across this membrane by at least two mechanisms: one Na^+-dependent and the other Na^+-independent. Both transport systems differ from the betaine transporters previously described by other authors in renal epithelium. The purpose of the current work was to characterize renal betaine transport using BBMV isolated from either the cortex or the medulla of rat kidney.

2. Materials and methods

2.1. Chemicals

[^{14}C]-betaine was purchased from GE Healthcare Europe, Gmbh; anti-OCTN3 (OCTN31-A) and anti-OCTN2 (OCTN21-A) antibodies from Alpha Diagnostic, San Antonio, TX; anti-SIT1 antibody (XTRP3 H-73) from Santa Cruz Biotechnology, Inc. European Support Office, Germany, and anti-Na^+,K^+,ATPase α-1 antibody from Upstate Biotechnology (New York, NY). Anti-OCTN2 is a 17 amino acid peptide and anti-OCTN3 a 19 amino acid peptide of mouse cytoplasmic C-terminus. Anti-SIT1 is a rabbit polyclonal antibody raised against amino acids 301–373 mapping within an extracellular domain of XTRP3 of human origin. Unless otherwise indicated the other reagents used in the current study were obtained from Sigma Chemical Co Madrid Spain.

2.2. Animals

Male Wistar rats (250 to 300 g) were sacrificed with a lethal intra-peritoneal injection of pentobarbital (50 mg/kg). The experiments were performed in accordance with national/local ethical guidelines.

2.3. BBMV preparation

Rat kidneys were excised, the renal capsules removed and the cortex manually separated from the medulla with a scalpel under magnifying lenses. BBMV were obtained following the method of Biber et al. [16]. Briefly, either kidney cortex or medulla slices from one rat were homogenized in 15 ml isolation buffer (in mM, 300 mannitol, 5 EGTA, 12 Tris–HCl, pH 7.4) with the Ystral Polytron on setting 5 for 2 min. $MgCl_2$, up to a final concentration of 10 mM, was added to the homogenate. The suspension was gently stirred for 20 min and then centrifuged at 1900 ×*g* for 15 min. The resultant supernatant was centrifuged at 30,000 ×*g* for 30 min and the resultant pellet was resuspended in 30 ml of 150 mM mannitol, 6 mM Tris–HCl (pH 7.4), 2.5 mM EGTA and homogenized with a glass-Teflon potter. $MgCl_2$, at a final concentration of 10 mM, was added to the homogenate. The suspension was gently stirred for 20 min and then centrifuged at 1900 ×*g* for 10 min. The resultant supernatant was centrifuged at 30,000 ×*g* for 30 min and the resultant pellet was resuspended in 0.5 ml of the appropriated loading buffer. Unless otherwise stated, the loading buffer consisted of (in mM) 140 mannitol, 50 Kgluconate and 50 Hepes–Tris (pH = 7.5). The suspension was made homogeneous by passing it through a 20-gauge needle several times and diluted up to 30 ml of the loading buffer. The suspension was centrifuged at 30,000 ×*g* for 30 min. The resultant pellet was resuspended in a selected volume of loading buffer and the isolated apical membranes were made homogeneous by passing them through a 25- and a 28-gauge needle several times and stored in liquid nitrogen until use. All the steps were carried out at 4 °C. When antibodies were used, the loading buffer contained the desired antibody and, before starting the uptake measurements, the vesicles pre-loaded with the antibody were incubated with it for 30 min at 37 °C.

Protein was measured by the method of Bradford [17] using γ-globulin as the standard.

The quality of the BBMV was evaluated by measuring a marker characteristic of apical membrane, alkaline phosphatase, and of basolateral membrane, Na^+/K^+/ATPase. Alkaline phosphatase activity was measured at 25 °C using alkaline phosphatase reagent following the manufacturer's instructions (Sigma Procedure No. 245). The amount of Na^+/K^+/ATPase present in the homogenates and BBMV was evaluated by Western blot using anti- Na^+/K^+/ATPase α-1 antibody, 1:100 dilution, as described [18]. Enrichment factors were calculated by reference to the homogenate. Alkaline phosphatase enrichment factors were 19- and 25-fold in cortex and medulla, respectively and that for Na^+/K^+/ATPase was 0.9 in both renal preparations.

2.4. Betaine uptake experiments

[^{14}C]-betaine uptake was measured at 37 °C as described [19]. Briefly, BBMV were left to stand at 37 °C for 10 min. Time incubations were initiated by adding 10 μl (250–300 μg of protein) of membrane vesicles suspension to 90 μl of uptake buffer. After designated periods of time, uptake was terminated by the addition of 1 ml of an ice-cold stop solution of the same composition as that of the intravesicular buffer. The samples were immediately filtered under vacuum through a 0.45 μm-pore size Millipore filter prewetted with the stop buffer. Filters were further washed twice with 5 ml of ice-cold stop solution and then dissolved in 5 ml of Ready-Protein (Beckman) scintillation fluid. The radioactivity was determined by liquid scintillation spectrometry. Nonspecific isotope binding to the filters was evaluated separately by adding stop solution to the vesicles before addition of uptake buffer and it was subtracted from the total radioactivity of each sample. The uptake buffer contained in mM: 140 mannitol, 50 NaCl, 50 HEPES–Tris (pH 7.5), 0.02 valinomycin and 0.01 [^{14}C]-betaine. When required, Kgluconate replaced NaCl. Valinomycin was dissolved in DMSO (0.1% v/v) and the vehicle alone had no effect on betaine transport measurements. All the measurements were done in triplicate.

2.5. Statistical analysis

Data are presented as mean ± SEM for (n) separated animals. In the Figures, the vertical bars that represent the SEM are absent when they are less than symbol height. Comparisons between different experimental groups were evaluated by the two-tailed Student's t-test. Two-way ANOVA followed by Newman–Keuls' test was used for multiple comparisons (GraphPad Prism Programme). Differences were set to be significant for $p < 0.05$.

3. Results

3.1. Binding vs. transport of [^{14}C]-betaine in cortical and medullar BBMV from rat kidney

When membrane vesicles are used, the nonspecific binding of the substrate to the vesicle surface should be determined to avoid overestimation of the solute transport into the vesicle. The nonspecific binding of betaine to the membrane is calculated from its vesicle uptake at infinite osmolarity and after 120 min incubation period. Fig. 1 shows that increasing osmolarity decreased betaine uptake, indicating that betaine is taken up into an osmotically sensitive vesicular space. The relationship between uptake and the reciprocal of osmolarity was fitted to a straight line and the intercept on the ordinate (zero intravesicular volume) is an estimation of nonspecific binding. The binding of betaine measured in cortical and medullar BBMV, both in the presence and in the absence of Na^+, represents in all cases less than 20% of the betaine associated with the vesicles under standard conditions.

From the known extravesicular betaine concentration, and assuming an equilibrium distribution of betaine at 120 min, the intravesicular volume was calculated. Under standard conditions, 300 mosM, the intravesicular volume of cortical and medullar BBMV was 1.2 ± 0.1 μl/mg protein.

3.2. Time course of betaine uptake into renal BBMV

[^{14}C]-betaine uptake into either cortical or medullar BBMV was measured both, in the presence and in the nominal absence of an inwardly-directed electrochemical NaCl gradient and as a function of time. An inwardly-directed NaCl chemical gradient was created by adding NaCl to the incubation buffer, being the intravesicular buffer nominally NaCl-free. An inside negative electrical membrane potential was created by an outwardly directed K^+ gradient in the presence of valinomycin. When required, the voltage across the membrane was clamped at zero by equal internal and external K^+ concentrations in the presence of valinomycin.

The time course of betaine uptake into renal BBMV revealed that betaine transiently accumulated in the vesicular space only in the presence of an inwardly directed electrochemical NaCl gradient (Fig. 2). The peak of uptake was reached at 30 s and it was significantly higher in the BBMV isolated from the cortex than in those from the medulla. No overshoot was observed when the extravesicular NaCl was replaced by isosmolar Kgluconate. The Na^+-independent betaine uptake was also significantly higher in cortex than in medulla.

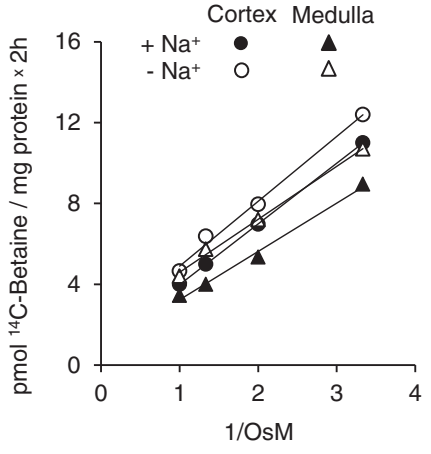

Fig. 1. External osmolarity and betaine uptake into BBMV isolated from either renal cortex or medulla. Medium osmolarity was increased by the addition of mannitol. 10 μM [^{14}C]-betaine uptake was measured during 120 min with or without extravesicular Na^+. The composition of the buffers is given in Materials and methods. Line was calculated by linear regression analysis ($r^2 > 0.999$). Each point represents the mean ± SEM of 4 separate BBMV preparations.

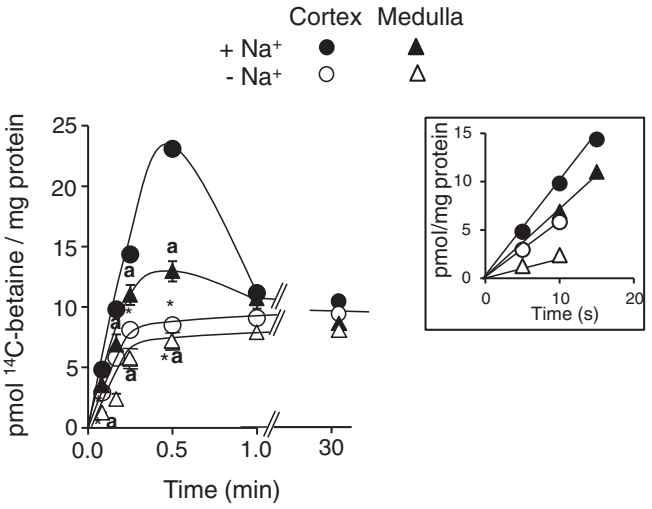

Fig. 2. Time-course of betaine uptake into BBMV isolated from rat renal cortex and medulla. 10 μM [^{14}C]-betaine uptake was measured in the absence and presence of inwardly directed Na^+ gradient as a function of time. Na^+ was isosmotically replaced by K^+. Each point represents the mean ± SEM of 5 separate BBMV preparations. Inset: initial rate of betaine uptake. One-way ANOVA showed an effect of Na^+ and of renal region on betaine uptake ($p < 0.001$). Newman–Keuls' test: *$p < 0.001$ without Na^+ *vs.* plus Na^+, [a]$p < 0.001$ medulla *vs.* cortex.

Fig. 2 inset reveals that betaine uptake increased linearly up to 15 s in the presence of NaCl and up to 10 s in Na^+-free conditions. On the bases of these results, a 5 s incubation time period was adopted to determine the initial rate of betaine uptake into the vesicles.

3.3. Ionic and electrical membrane potential dependence of betaine uptake into BBMV

The following set of experiments were undertaken to determine the ionic dependence of the transporters that carry betaine into the renal BBMV obtained from either the cortex or medulla. Initial rate of betaine uptake was measured in the presence and absence of either electrical membrane potential, Na^+ or NaCl gradients. NaCl chemical gradient or electrical membrane potential was created or abolished as mentioned above. Na^+ chemical gradient/Cl^--free conditions were created by isosmotic replacement of NaCl with Nagluconate. In all cases the intravesicular buffer was nominally free of NaCl and contained 50 mM Kgluconate.

The results (Fig. 3) reveal that in the presence of a chemical NaCl gradient, clamping the membrane voltage at zero inhibited betaine uptake and either NaCl- or Cl^--free conditions further increased this inhibition. The inhibition induced by the absence of Cl^- was not significantly different from that measured in the absence of NaCl and membrane voltage had no effect on betaine uptake under these conditions. Together these observations indicate that rat renal BBMV present a betaine transport system that is electrogenic and requires the presence of both, Na^+ and Cl^- in the extravesicular buffer.

3.4. Effect of organic compounds on betaine uptake into renal BBMV

To further characterize betaine transport in rat renal BBMV we tested the effect of organic compounds on betaine uptake into the vesicles. Initial rate of [^{14}C]-betaine uptake was measured in cortical and medullar BBMV in the presence and absence of an inwardly directed electrochemical NaCl gradient and in the presence and absence of cold betaine and other organic compounds. Only a few inhibitors were proved with BBMV isolated from the renal medulla because the betaine transport activity was less than half of that measured in cortical vesicles. Table 1

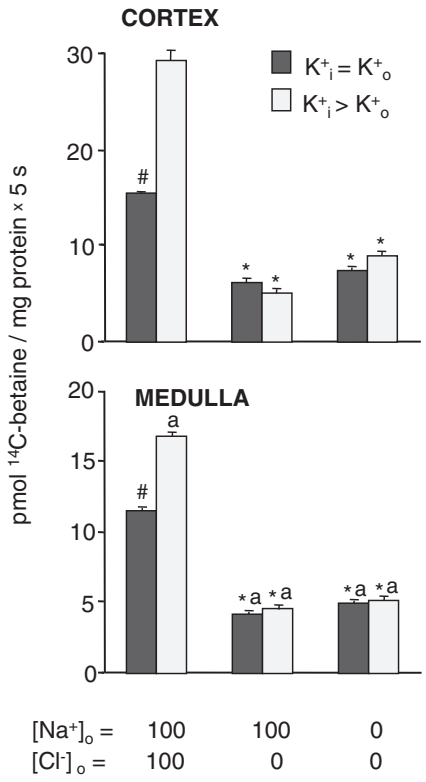

Fig. 3. Effect of sodium chloride, chloride and electrical membrane potential on betaine uptake into rat renal BBMV. 10 μM [^{14}C]-betaine uptake was measured in the absence and presence of electrical membrane potential and/or either NaCl or Cl^- chemical gradient. The buffer composition is given in Materials and methods. Membrane potential was generated by an outwardly directed K^+ gradient plus 20 μM valinomycin and it was abolished by equal intra and extravesicular K^+ concentration plus 20 μM valinomycin. When required, Cl^- was substituted by equimolar concentration of gluconate and NaCl by mannitol. The time of uptake was 5 s. Each point represents the mean ± SEM of 5 separate BBMV preparations. One-way ANOVA showed an effect of NaCl, Cl^- and electrical potential and of renal region on betaine uptake ($p < 0.001$). Newman–Keuls' test: $^*p < 0.001$ *vs*. NaCl gradient; $^\#p < 0.001$ *vs*. electrical membrane potential; $^ap < 0.001$ medulla *vs*. cortex.

shows that in both, cortex and medulla the most potent inhibitor of the NaCl-dependent betaine uptake was L-proline (75% inhibition), followed by cold betaine and L-carnitine (50% inhibition) and by choline (40–60% inhibition). Betaine uptake was unaffected either by TEA, creatine, GABA, β-alanine, glycine or taurine.

Betaine uptake measured under nominal NaCl-free conditions was also inhibited by some of the organic compounds tested. The more potent inhibitor was cold betaine, which nearly prevented betaine transport (approx. 95% inhibition), followed by L-carnitine (70–80% inhibition) and proline (40–50% inhibition). Contrary to what was observed in the presence of NaCl, NaCl-independent betaine transport was inhibited by TEA (approx. 50% inhibition) whereas choline had no effect. The other organic compounds tested inhibited Na^+-independent betaine uptake by about 10 to 20%.

The different sensitivity of the NaCl-dependent and NaCl-independent betaine transport to inhibition by organic compounds corroborates the hypothesis that two different transporters mediate betaine uptake across the apical membrane of renal epithelium.

Table 1
Specificity of betaine transport in renal BBVM.

	Relative betaine transport rates (% of control)			
	Na^+-dependent		Na^+-independent	
	Cortex	Medulla	Cortex	Medulla
Control	100 ± 0.1	100 ± 4	100 ± 5	100 ± 8
L-proline	26.0 ± 3*	23.9 ± 5*	50 ± 4*	64 ± 5*
Betaine	47.9 ± 4*	42.6 ± 2*	7.3 ± 1*	6.5 ± 2*
L-carnitine	46.3 ± 2*	31.3 ± 5*	15.4 ± 1.4*	26 ± 4*
Creatine	86 ± 4	93 ± 8	71 ± 4	85 ± 7
Choline	62 ± 12*	41 ± 8*	111 ± 4	115 ± 2
TEA	94 ± 14	124 ± 3	44 ± 16*	54 ± 7*
Taurine	114 ± 14		85 ± 5	
β-alanine	120 ± 20		74 ± 4	
GABA	108 ± 8		79 ± 6	
Glycine	100 ± 5		83 ± 6	

10 μM [^{14}C]-betaine uptake was measured for 5 s in the presence and absence of the indicated modifiers and with or without Na^+ in the extravesicular buffer. The concentration of the modifiers was 5 mM. Na^+-dependent uptake: uptake measured in the presence of Na^+ minus that in its absence. The uptake measured in the absence of modifiers was set at 100% (control). Means ± SEM of 4 independent experiments. $^*p < 0.001$ as compared with control.

3.5. pH and Na^+-independent betaine uptake in BBMV from the renal cortex

As a Na^+-independent, pH-dependent uptake of betaine had been previously detected at the apical membrane of rabbit renal epithelium [10], we looked at the effect of pH on the Na^+-independent betaine transport in BBMV from the rat renal cortex. The results revealed that neither an inside directed (5.2 ± 0.2 pmol betaine/mg protein/5 s at pH_i 7.5/pH_o 6 *vs.* 5.4 ± 0.3 with pH_i 7.5/pH_o 7.5) nor an outside directed (4.9 ± 0.2 pmol betaine/mg protein/5 s at pH_i 6/pH_o 7.5 *vs*. 4.7 ± 0.5 with pH_i 6/pH_o 6) H^+ gradient affected Na^+-independent betaine uptake, indicating that apical Na^+-independent betaine transport in rat kidney is neither co-transported nor exchanged with H^+.

3.6. Kinetics of the renal transport of betaine

We next evaluated the apparent maximal initial rates of uptake, V_{max}, and the Michaelis–Menten constant, K_t, for the NaCl-dependent and NaCl-independent betaine transport systems. Cortical BBMV were used for the reasons given above. 5 s betaine uptake was measured over the extravesicular betaine concentration range of 0.01 to 5 mM in the presence and absence of NaCl and the results are given in Fig. 4.

Electrogenic NaCl-dependent betaine uptake is the difference between uptakes measured in the presence and absence of NaCl (Fig. 4a). The analysis of the data with a non-linear regression analysis programme (ENZFITTER) revealed that the NaCl-dependent betaine uptake fit best ($r = 0.99$) a transport model describing a single saturable transport system:

$$v = V_{max}S/(K_t + S)$$

and that the uptake measured under NaCl-free conditions fit best ($r = 0.96$) a transport model describing a single saturable transport system plus a nonsaturable diffusion component:

$$v = V_{max}S/(K_t + S) + SK_D$$

where v, is the initial rate of uptake; S, the extravesicular betaine concentration, and K_D the diffusion component.

The K_D has a value of 0.18 ± 0.01 pmol/mg protein/s/mM and it was used to estimate the non-carrier mediated diffusion component. The data shown in Fig. 4b are the difference between the betaine uptake measured under NaCl-free conditions minus the calculated diffusion component. The Eadie–Hofstee plot of the saturable components (Fig. 4, insets) yield in both cases a linear relationship, consistent with the existence in each case of a single saturable betaine transporter. The calculated apparent K_t and V_{max} values were 1.1 ± 0.5 mM and 498 ± 98 pmol/mg protein/s, respectively, for the NaCl-dependent and 47 ± 7 μM and 7.8 ± 1 pmol/mg protein/s, respectively, for the NaCl-independent betaine transport systems.

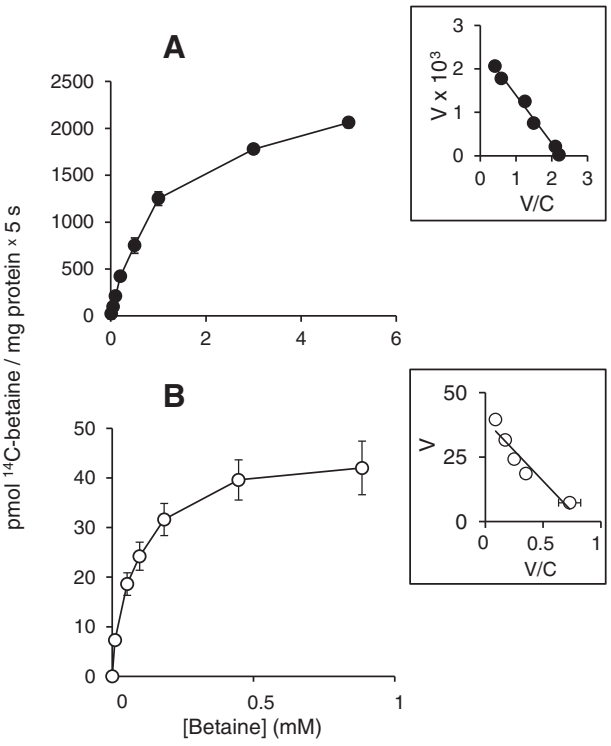

Fig. 4. Na^+-dependent and Na^+-independent betaine uptake rate *vs.* increasing concentrations of extravesicular betaine. The external betaine concentration ranged from 0.01 mM to 5 mM. The time of uptake was 5 s. A. NaCl-dependent transport was calculated from uptake measured in the presence of NaCl minus that obtained in its absence. B. Uptake measured in the nominal absence of NaCl minus the calculated diffusion component. Each point represents the mean ± SEM of 5 separate BBMV preparations. Inset: Eadie–Hofstee plots of the data.

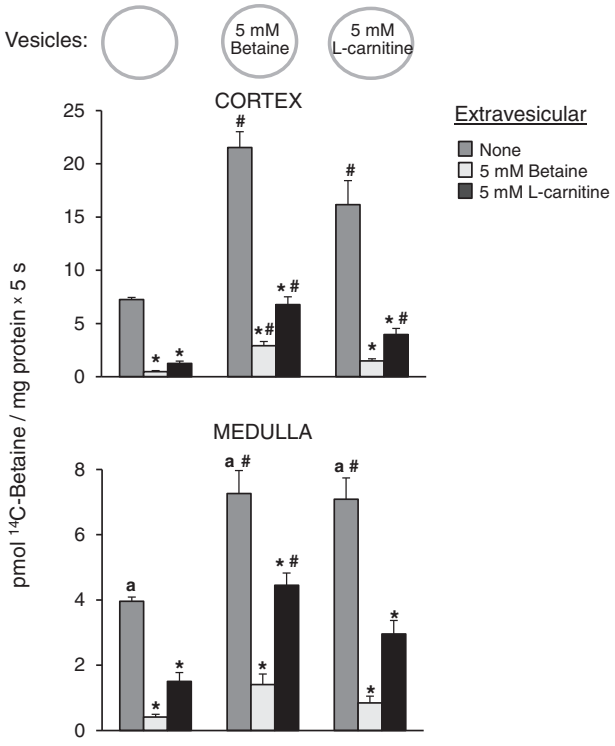

Fig. 5. Trans-effect of either betaine or L-carnitine on Na^+-independent betaine uptake. BBMV from both, cortex and medulla were either unloaded or preloaded with 5 mM of either betaine or L-carnitine. 10 μM [^{14}C]-betaine uptake was measured in the presence and absence of either 5 mM betaine or 5 mM L-carnitine in the extravesicular buffer. The time of uptake was 5 s. The extravesicular buffer was nominally NaCl-free. Each point represents the mean ± SEM of 5 separate BBMV preparations. One-way ANOVA showed an effect of intravesicular betaine or L-carnitine, of extravesicular betaine or L-carnitine and of renal region on betaine uptake ($p < 0.001$). Newman–Keuls' test: $^{\#}p < 0.001$ loaded *vs.* unloaded vesicles; $^{*}p < 0.001$ extravesicular inhibitors *vs.* its absence; $^{a}p < 0.001$ medulla *vs.* cortex.

3.7. Trans-effect of betaine and L-carnitine on Na^+-independent betaine uptake rate

The results described so far indicate that betaine uptake into the renal BBMV is in part mediated by a Na^+-, Cl^-- and H^+-independent transport system. We next determined whether this betaine transporter functions as an exchanger of betaine with other substrates. For these experiments some BBMV were preloaded with 5 mM of either betaine or L-carnitine. These substrates were chosen because they are the more potent inhibitors of NaCl-independent betaine uptake. 5 s betaine uptake into either unloaded or loaded vesicles was measured under NaCl-free conditions and in the presence and absence of extravesicular betaine (5 mM) or L-carnitine (5 mM).

Fig. 5 shows that intravesicular betaine or L-carnitine stimulated betaine uptake into the BBMV being the trans-stimulation higher in the BBMV obtained from the cortex than in those from the medulla. That either betaine or L-carnitine accelerated exchange diffusion (trans-stimulation) of betaine across the membrane vesicle, indicated that the underlying carrier can function as an exchanger of betaine/betaine or betaine/L-carnitine. In all the experimental conditions tested betaine uptake into the vesicles was inhibited by extravesicular betaine or L-carnitine, being the inhibition induced by betaine higher than that produced by L-carnitine. These observations support the view that the transporter has higher affinity for betaine than for L-carnitine.

3.8. Effect of anti-OCTN2, anti-OCTN3 and anti-SIT1 antibodies on betaine transport in BBMV of rat renal cortex

As the Na^+-independent betaine transport system is inhibited by L-carnitine and exchanges betaine/L-carnitine, the following experiments were designed to determine whether the L-carnitine transporters OCTN2 (Na^+-dependent) and OCTN3 (Na^+-independent) mediate betaine uptake under Na^+-free conditions. The involvement of OCTN2 was tested because under Na^+-free conditions it transports L-carnitine with low affinity [20]. We measured the effect of anti-OCTN2 and anti-OCTN3 antibodies on betaine uptake into cortical BBMV under NaCl-free conditions. Four types of vesicles were used: unloaded vesicles, loaded with 5 mM L-carnitine, loaded with 5 mM L-carnitine plus anti-OCTN2 antibody and loaded with 5 mM L-carnitine plus anti-OCTN3 antibody. The results (Fig. 6) show that neither anti-OCTN3 nor anti-OCTN2 antibodies significantly affected betaine uptake into L-carnitine-preloaded BBMV.

To discard the possibility that the Na^+-independent betaine transport system is a different modality of SIT1 working in Na^+-free conditions, we tested the effect of anti-SIT1 antibody on betaine uptake into cortical BBMV. The results (Fig. 7) reveal that whereas anti-SIT1 antibody blocks Na^+-dependent betaine transport activity it had no effect on the betaine/betaine exchange.

4. Discussion

Betaine is rapidly absorbed by the small intestine and efficiently reabsorbed by the kidney even at relatively high doses of betaine in serum [6,7]. Secondary Na^+- and/or Cl^--coupled and Na^+-independent betaine transport systems have been described in intestinal and renal epithelia and in organisms from bacteria to vertebrates [see 11,21,22 for revisions]. Here we present evidence for the presence at the apical membrane of rat renal epithelium of two betaine transport systems, which differ in their Na^+ and Cl^- dependence, electrogenicity, ability to

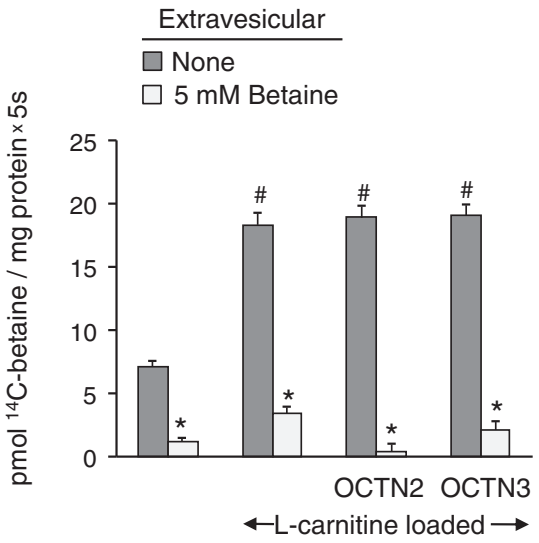

Fig. 6. Effect of anti-OCTN3 and anti-OCTN2 antibodies on Na^+-independent betaine uptake. BBMV obtained from the rat renal cortex were either unloaded or preloaded with 5 mM L-carnitine and the latter without or with either anti-OCTN3 or anti-OCTN2 antibody (diluted 1:10). 10 μM [^{14}C]-betaine uptake was measured for 5 s in the nominal absence of NaCl and with or without extravesicular 5 mM cold betaine. Each point represents the mean ± SEM of 5 separate BBMV preparations. One-way ANOVA showed an effect of intravesicular L-carnitine and of extravesicular betaine on betaine uptake ($p < 0.001$). Newman–Keuls' test: $^{\#}p < 0.001$ L-carnitine loaded *vs.* unloaded vesicles; $^{*}p < 0.001$ with *vs.* without extravesicular cold betaine.

concentrate betaine into the BBMV space, sensitivity to some organic substrates and apparent K_t and V_{max} for betaine. The same two betaine transporters are present in the cortex and in the medulla because in both renal regions they are inhibited by the same compounds and with the same order of potency. Their transport capacities, however, are higher in the cortex than in the medulla, suggesting higher number of transporters in the former than in the latter renal region.

Previous studies showed that the Na^+-dependent betaine uptake into BBMV isolated from rabbit kidney was Cl^--independent [10], but

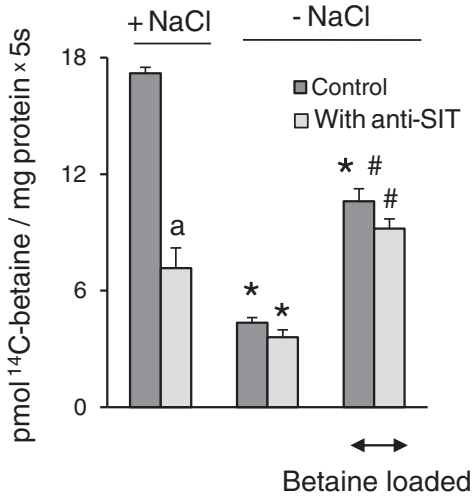

Fig. 7. Effect of anti-SIT1 antibody on Na^+-dependent and Na^+-independent betaine uptake. 10 μM [^{14}C]-betaine uptake into cortical BBMV was measured for 5 s in the presence and absence of NaCl in the incubation buffer and in the presence and absence of anti-SIT1 antibody. BBMV were either unloaded or preloaded with 5 mM betaine and the latter without or with anti-SIT1 antibody (diluted 1:10). Each point represents the mean ± SEM of 5 separate BBMV preparations. One-way ANOVA showed an effect of intravesicular betaine on betaine uptake ($p < 0.001$). Newman–Keuls' test: *with *vs.* without Na^+; $^{\#}p < 0.001$ betaine loaded *vs.* unloaded vesicles; $^{a}p < 0.001$ without *vs.* with anti-SIT1 antibody.

the current work reveals that, in both, the cortex and medulla of rat kidney, the apical Na^+-dependent betaine transport requires external Cl^-. The stoichiometry of the transporter has not been evaluated in the current study, but its electrogenicity suggests that at least 2 Na^+ and 1 Cl^- are required to transport 1 betaine molecule. Our observation agrees with that of Grunewald et al. [23] who measured in the thick ascending limb of Henle's loop culture cells an apical membrane Na^+/Cl^-/betaine cotransport activity. They proposed that this transport activity is mediated by BGT1, a transporter normally localized at the basolateral membrane of renal epithelium. BGT1 does not appear to mediate the apical Na^+/Cl^-/betaine transport described in the current work because BGT1: i) is predominantly expressed in the medulla [24], ii) has higher affinity for GABA than for betaine [2,3] and iii) has very low affinity for proline [2], but the Na^+/Cl^-/betaine transport activity measured here is greater in the cortex than in the medulla, unaffected by GABA and inhibited by L-proline (around 75% inhibition) to greater extent than by betaine (50% inhibition). The observation that the Na^+/Cl^-/betaine transport activity is prevented by anti-SIT1 antibody indicates that the transporter involved is the SIT1/XT3s1, which in oocytes functions as a Na^+- and Cl^--dependent proline-preferring imino acid transporter [14]. The affinity constant for betaine ($K_t = 1.1$ mM) of the Na^+/Cl^-/betaine transporter described here is similar to that measured by Pummer et al. [9] in descending limbs of short Henle's loops (1.2–1.6 mM) but higher than that measured for SIT1 ($K_t = 200$ μM) in oocytes [13]. These differences could be due to the preparation used (intact kidney and BBMV *vs.* oocytes). Although SIT1 had been detected at the apical membrane of the human and mouse proximal tubule by immunohistochemistry [25,15], as far as we are aware this is the first work reporting a functional SIT1 that mediates betaine uptake across the apical membrane of renal epithelium.

The current study also reveals that in the nominal absence of NaCl, betaine uptake into rat renal BBMV was inhibited by several organic compounds and followed Michaelis–Menten kinetics indicating that this uptake is carrier-mediated. Additional experiments revealed that the transporter functions as an antiporter: betaine uptake was transstimulated by intravesicular betaine or L-carnitine, indicating that the influx of betaine and the efflux of either betaine or carnitine are linked. Wunz and Wright [10] detected in rabbit renal BBMV a Na^+-independent betaine transport activity that was stimulated by H^+ gradient, blocked by glycine and reached half-maximal uptake at a betaine concentration, K_t, of 2.8 mM. This transport system is currently known as PAT [see 11 for a review]. However, the Na^+-independent betaine transport activity described here is H^+-independent, not significantly inhibited by glycine and its K_t for betaine is 47 μM. Explanations for why we did not measure a H^+-sensitive betaine transport activity could be that: i) although PAT1 mRNA has been detected in all rat, mouse and human tissues studied, it is localized mainly in lysosomes and involved in H^+-coupled amino acid transport in this compartment [26,27] and ii) there are species differences in the pH-dependency and tissue expression of the amino acid transporters [11].

The results discussed so far indicate that the Na^+-independent transport activity measured here is carrier-mediated, but the carrier involved is unknown. Several observations favour the view that this unknown carrier is an entity different from that mediating Na^+/Cl^-/betaine transport. First, the sensitivity of the Na^+-independent betaine transport to inhibition by organic compounds differs from that of the Na^+/Cl^-/betaine transporter above described: i) cold betaine, and no proline, is its more potent inhibitor, ii) is inhibited by TEA, that has no effect on the Na^+/Cl^-/betaine transport, and iii) is unaffected by choline, an inhibitor of the Na^+/Cl^-/betaine transport. Secondly, the affinity of the Na^+-dependent transporters for the substrate, e.g. carnitine, decreases in the absence of Na^+ [20] but the affinity for betaine of the Na^+-independent transporter (47 ± 7 μM) is greater than that of the Na^+/Cl^-/betaine transporter (1.1 ± 0.5 mM). And third, the anti-SIT1 antibody had no effect on the Na^+-independent transport activity but prevented Na^+/Cl^-/betaine transport.

We previously reported that OCTN3 transporter is present at the apical membrane of rat renal epithelium and can exchange L-carnitine/L-carnitine or L-carnitine/betaine [17]. However, OCTN3 does not appear to mediate the Na^+-independent betaine uptake because it is a L-carnitine preferring transporter and the one describing here is more sensitive to inhibition by betaine than to L-carnitine. In addition, OCTN3 exchanger activity is inhibited by anti-OCTN3 antibody [17] and neither this antibody nor an anti-OCTN2 antibody inhibited the betaine/L-carnitine exchange described here. Tsuchida et al. [28] identified, at the apical membrane of mice proximal tubules, a Na^+- and H^+-independent L-carnitine transporter (OAT9) that is inhibited by betaine, but OAT9 resembles the OCTN3 identified by us at the apical membrane of rat renal epithelia [17] in that both are more sensitive to inhibition by L-carnitine than to betaine and have similar K_t for L-carnitine (OAT9, 2.9 μM and OCTN3, 7.3 μM).

The physiological role of co-expression of two different transporters for the same substrate at the apical membrane of renal epithelium is unknown but not infrequent. With very few exceptions, individual amino acids are transported by more than one transport system that could serve to provide backup capacity for absorption in the case of mutational inactivation of a transport system [see 29 for a review]. In addition, the amino acid transporters present a structural fold, the APC superfamily fold, which is shared by prokaryotes and eukaryotes. The APC fold provides different possibilities to transport the same amino acid, so that each amino acid transport system adapts to the environmental conditions by choosing the coupling mode that allows to achieve the affinity required for certain physiological conditions [29–31]. For instance, the bacterial betaine–choline–carnitine transporter family includes Na^+- and H^+-coupled symporters as well as antiporters [22]. The Na^+/Cl^-/betaine transporter described here could mediate betaine transport at luminal betaine concentrations in the mM range, whereas the Na^+-independent transporter might become more important at μM betaine concentrations. It is relevant that the human serum levels of betaine range from 30 to 47 μM, values close to the K_t of the transporter measured here. The Na^+-independent transporter could also contribute to the excretion of cationic drugs and other xenobiotics in exchange with tubular betaine or even to betaine excretion from medullar cells that have previously accumulated betaine under hyperosmotic shock. It is interesting to point out that the exchanger described here and the OCTN3 exchanger previously described [17] resemble the bacterial CaiT. The CaiT allows bacteria to take up L-carnitine under anaerobic growth conditions and convert it first into crotonobetaine (an intermediate that probably serves as an electron acceptor) and then into gamma-butyrobetaine, the excreted end product of the pathway

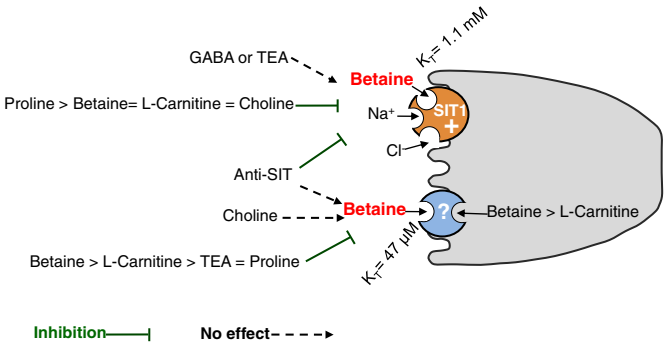

Fig. 8. Betaine transport at the apical membrane of rat renal epithelium. The results suggest that betaine crosses the apical membrane of rat renal epithelium via two transporters. One is Na^+- and Cl^--dependent, electrogenic (+) and inhibited by anti-SIT1 antibody (SIT1). The other is Na^+- and H^+-independent, unaffected by anti-SIT1 antibody and it exchanges betaine for either betaine or L-carnitine. Both transporters also differ in their affinity (K_T) for betaine and sensitivity to inhibitors.

[32]. Whether OCTN3 and the betaine antiporter described here are involved in renal metabolic reactions is unknown.

In conclusion, we present evidence indicating that betaine crosses the apical membrane of rat renal epithelia via SIT1 transporter and via a non-concentrative, Na^+- and H^+-independent transporter (see Fig. 8), which functions as an exchanger and resembles OCTN3 and the bacterial CaiT. The kinetic properties of both transporters favour their involvement in the efficient tubular reabsorption of the betaine filtered by the glomeruli. As far as we know this is the first work reporting that betaine crosses the apical membrane of rat renal epithelium by both, a Na^+- and H^+-independent transport system and by a transporter that requires the presence of both, Na^+ and Cl^- in the incubation buffer.

Conflict of interest

The authors disclose no conflict of interests.

Acknowledgements

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