Stoichiometric studies of β -alanine transporters in rabbit proximal tubule

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The coupling ratio for the transport of β -alanine and Na^+ , H^+ and Cl^- in luminal membrane vesicles isolated from proximal convoluted tubules (pars convoluta) and proximal straight tubules (pars recta) of rabbit kidney was examined. Indirect evidence indicates that 1 H^+ and approx. 2 Na^+ , 1 Cl^- (Na^+ -dependent, high-affinity) or 1 Na^+ (Na^+ -dependent, low-affinity) are co-transported with β -alanine in the pars convoluta. In pars recta, the two Na^+ -dependent transporters exhibited the same stoichiometric properties respectively as in pars convoluta.

INTRODUCTION

A number of studies have indicated that accumulative transport of β -alanine in renal brush-border membrane vesicles from rabbit whole cortex (Hammerman & Sacktor, 1978) and dog outer cortex (Turner, 1986) is dependent on the presence of an Na^+ concentration gradient. Kinetic analysis has suggested the existence of a single high-affinity transport system that is specific for β -amino acids. Furthermore, it was observed that the Na^+ -dependent uptake of β -alanine is specifically stimulated by Cl^- and could be driven against a concentration gradient by a Cl^- gradient. The transport of β -alanine was demonstrated to be an electrogenic process, and estimations of the coupling ratio suggested approx. 2 Na^+ :1 Cl^- :1 β -alanine. However, we have recently demonstrated the existence of both H^+ - and Na^+ -dependent multiple transport systems for β -alanine in luminal membrane vesicles from rabbit proximal tubule (for details, see Jessen *et al.*, 1989). In the present paper the determinations of the stoichiometric coupling ratio between flows of cations, anions and β -alanine are investigated, also with regard to the different segmental localization of the transport systems in the proximal tubule.

EXPERIMENTAL

Preparation of luminal membrane vesicles

Luminal membrane vesicles were isolated from pars convoluta ('outer cortex') and from pars recta ('outer medulla') of the proximal tubules of rabbit kidney according to methods already described (Sheikh & Møller, 1987) and mentioned here only briefly. The renal tissue was homogenized and luminal membrane vesicles were prepared by differential centrifugation and Ca^{2+} precipitation. The purity of the membrane vesicle preparation with regard to the content of luminal vesicles was examined by electron microscopy (Kragh-Hansen *et al.*, 1985) and by measuring specific activities of various enzyme markers (Sheikh *et al.*, 1982). The activity of alkaline phosphatase in the luminal-membrane-vesicle fractions was enriched as compared with the corresponding homogenates by the following factors ($n = 15$): 9.4 ± 1.8 -fold (pars convoluta) and 11.2 ± 2.6 -fold (pars recta). For leucine aminopeptidase the enrichment factors were 17.5 ± 2.7 -fold (pars convoluta) and 24.7 ± 3.6 -fold (pars recta), and maltase was enriched 9.4 ± 2.4 -fold (pars convoluta) and 8.6 ± 2.3 -fold (pars recta). Average enrichments in specific activity of the basolateral marker Na^+ + K^+ -stimulated ATPase, and that of the mitochondrial marker succinate dehydrogenase, were in all cases < 0.4 . The amount of protein was determined by the method of Lowry *et al.* (1951), modified as described by Peterson

(1977), with BSA (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Uptake of β -alanine by membrane vesicles

The uptake of β -alanine by luminal membrane vesicles was studied by Millipore filtration (Hopfer *et al.*, 1973). The details of the individual experiments are given in legends to the Figures. In a series of experiments in which the Na^+ -coupling ratio with β -alanine was investigated, the transmembrane electrical potential differences were minimized by short-circuiting the membrane. Examining the stoichiometry of the Na^+ - β -alanine co-transport mechanism for Cl^- , vesicles were preincubated with potassium gluconate and valinomycin in order to clamp membrane potentials at the K^+ diffusion potential. The time point at which the fluxes were measured was 20 s. The fractional uptakes relative to the maximal and the equilibrium uptake were in pars convoluta and pars recta 0.42 ± 0.06 , 1.62 ± 0.19 and 0.39 ± 0.05 , 1.80 ± 0.21 respectively. The reasons for studying the uptake of β -alanine at 20 s incubation time are partly that the radioactive- β -alanine uptake is reduced, short-circuiting the electrical potential across the membrane, and partly that the radioactive- β -alanine uptake at increasing unlabelled- β -alanine concentrations in the medium is small below an incubation time of 20 s, resulting in very scattered results. The results given in the Figures are mean values for four experiments, performed in triplicate, the S.D. being approx. 10 %.

Calculations

In order to establish the transport stoichiometry between ion and β -alanine fluxes, the 'activation method' (Turner & Moran, 1982a) was applied to these vesicle preparations. Here one measures the stimulation of substrate (β -alanine) flux by increasing concentrations of activator (Na^+ , H^+ or Cl^-). The data were analysed as follows (Turner & Moran, 1982b):

$$\text{Flux} = V_{\text{max}} \cdot [\text{A}]^n / (K_{0.5}^n + [\text{A}]^n)$$

The equation assumes the existence of n essential co-operative site(s) for the activator, A, per β -alanine site. According to this equation, a plot of $\text{flux}/[\text{A}]^n$ against flux for the correction of n will yield a straight line with slope $1/K_{0.5}^n$.

RESULTS AND DISCUSSION

Kinetic studies of β -alanine uptake by luminal membrane vesicles

In our previous report (Jessen *et al.*, 1989) we showed that the Na^+ -conditional transport of β -alanine in vesicles from pars

Table 1. Cl⁻-specificity of β -alanine (β -Ala) uptake in luminal membrane vesicles from pars convoluta and pars recta

Vesicles were prepared in a solution containing 310 mM-mannitol and 15 mM-Hepes/Tris, pH 7.5. Measuring the Na⁺-dependent uptake of β -alanine, the incubation media contained 15 mM-Hepes/Tris, pH 7.5, 155 mM of one of the anions listed below, as the sodium salt, 350 nM- β -[3-³H(n)]alanine, and unlabelled β -alanine reaching the final concentration shown in the Table. Measuring the H⁺-dependent uptake of β -alanine, the experimental conditions were essentially the same as those just mentioned, except that Na⁺ was replaced by K⁺ and the incubation media contained 15 mM-Mes/Tris, pH 5.5, and 460 nM- β -[3-³H]alanine. Solute uptakes at 20 s were normalized to uptake observed in the presence of NaCl or KCl respectively. Results are given as means $\pm$ S.D. for five experiments.

Anion	Solute uptake relative to that in Cl ⁻ medium				
	Pars convoluta			Pars recta	
	Na ⁺ -dependent		H ⁺ -dependent	Na ⁺ -dependent	
	350 nM- β -Ala	3 mM- β -Ala	460 nM- β -Ala	350 nM- β -Ala	3 mM- β -Ala
Cl ⁻	1.00	1.00	1.00	1.00	1.00
NO ₃ ⁻	0.74 $\pm$ 0.11	1.19 $\pm$ 0.10	1.27 $\pm$ 0.14	0.37 $\pm$ 0.06	1.30 $\pm$ 0.14
Gluconate	0.32 $\pm$ 0.07	0.61 $\pm$ 0.07	0.23 $\pm$ 0.03	0.08 $\pm$ 0.07	0.60 $\pm$ 0.10

Fig. 1. Effect of Na⁺ concentration on the uptake of β -alanine by luminal membrane vesicles from pars convoluta

Vesicles were preincubated in 15 mM-Hepes/Tris buffer, pH 7.5, containing 100 mM-KSCN, 700 mM-mannitol and valinomycin at a concentration of 12.5 μ g/mg of protein. The incubation media contained 15 mM-Hepes/Tris/100 mM-KSCN/300 mM-mannitol and various concentrations of NaCl ranging from 0 to 200 mM (final concn.). Choline replaced Na⁺ iso-osmotically to obtain the various Na⁺ concentrations studied. The composition of the stop buffer was 15 mM-Hepes/Tris (pH 7.5)/400 mM-mannitol/300 mM-NaCl. All uptake values were corrected for uptake in the absence of a Na⁺ gradient (uptake_{0 mM-Na⁺} = 1.42 nmol/min per mg of protein). (a) Plot of flux versus Na⁺ concentration for 750 nM- β -[3-³H(n)]alanine and unlabelled β -alanine reaching a final concentration at 3 mM. The curve shows the difference between flux at 3 mM and 600 nM in order to examine only the low-affinity transport component. (b) Plots of flux/[Na⁺]ⁿ versus flux for n = 1 (●) and n = 2 (○) for the low-affinity system (3 mM minus 600 nM). The units of [Na⁺] are M.

Fig. 2. Effects of H⁺ gradient (extravesicular > intravesicular) on the uptake of β -alanine by luminal membrane vesicles from pars convoluta

The vesicles were preincubated with 155 mM-KCl in 15 mM-Hepes/Tris, pH 7.5. The incubation media contained 460 nM- β -[3-³H(n)]alanine and 155 mM-KCl in various Hepes/Tris or Mes/Tris buffer solutions with pH ranging from 7.5 to 5.0. All uptake values were corrected for the uptake in the absence of a H⁺ gradient (pH_{out} = pH_{in} = 7.5; uptake_{0 mM-H⁺} = 0.99 pmol/min per mg of protein). Plots of flux/[H⁺] versus flux for n = 1 (■) and n = 2 (□) are shown.

convoluta was mediated by a single transport component with a half-saturation constant of 2.66 mM. However, at the same time, Wolff & Kinne (1988) provided evidence for the existence of a very high-affinity transport system (K_m = 35 μ M) for taurine in rabbit renal outer-cortical brush-border membrane vesicles. Since β -alanine appears to be transported as taurine (i.e. a β -amino-acid-specific transport component), we searched for a very-high-affinity transport system for β -alanine in pars convoluta. By using β -[³H]alanine instead of β -[¹⁴C]alanine, it was possible to perform experiments at much lower concentrations of β -alanine. We observed an additional Na⁺-dependent high-affinity transport system. Thus, in pars convoluta, three transport systems are found: two Na⁺-dependent systems ($K_{m,1}$ = 0.31 mM, $K_{m,2}$ = 2.92 mM, $V_{max,1}$ = 0.55 nmol $\cdot$ 20 s⁻¹ $\cdot$ mg of protein⁻¹ and $V_{max,2}$ = 2.34 nmol $\cdot$ 20 s⁻¹ $\cdot$ mg of protein⁻¹), and an H⁺-dependent system (K_m = 4.03 mM and V_{max} = 2.43 nmol $\cdot$ 20 s⁻¹ $\cdot$ mg of protein⁻¹).

In pars recta the transport of β -alanine occurs via a dual transport system ($K_{m,1} = 0.13$ mM, $K_{m,2} = 8.86$ mM, $V_{max,1} = 1.46$ nmol $\cdot$ 20 s⁻¹ $\cdot$ mg of protein⁻¹ and $V_{max,2} = 2.81$ nmol $\cdot$ 20 s⁻¹ $\cdot$ mg of protein⁻¹) strictly dependent on Na⁺, as previously shown. Results are means for three experiments.

Cl⁻-specificity of β -alanine uptake

Cl⁻ replacement under conditions of a salt gradient (Table 1) revealed a specific anion-dependence for β -alanine transport for the Na⁺-dependent high-affinity transporter, in both pars convoluta and pars recta. Similar results (Cl⁻ > NO₃⁻ > gluconate) have been reported for β -alanine uptake in dog brush-border membrane vesicles from outer cortex (Turner, 1986). The other transport systems were found to be dependent on the different anion diffusion potentials (NO₃⁻ > Cl⁻ > gluconate), as previously described for the Cl⁻-independent glucose transport (Ullrich, 1979), indicating an electrogenic transport of β -alanine.

Stoichiometry of Na⁺, H⁺ and Cl⁻-dependent β -alanine transport in vesicles from pars convoluta

To assess the stoichiometry, we studied the dependence of β -alanine uptake on the extravesicular concentrations of Na⁺, H⁺ and Cl⁻. Since the overall transport of β -alanine with respect to Na⁺ depends on the existence of two transport systems with different half-saturation values, we investigated the coupling ratio of Na⁺ to β -alanine in vesicles from pars convoluta by measuring the initial flux of 600 nM- (high-affinity) and 3 mM- β -alanine (low-affinity) as a function of Na⁺ concentration over the range 0–200 mM. The concentration of Cl⁻ was maintained constant at 200 mM by replacing NaCl iso-osmotically with choline chloride in order to obtain the various Na⁺ concentrations studied. At 600 nM- β -alanine, the flux data showed a marked sigmoidal dependence on Na⁺ concentration, indicating that more than one Na⁺ ion is involved in the translocation process (results not shown). A value of $n = 1.8$ (see under 'Calculations' in the Experimental section) resulted in the best fit of the above-mentioned data, suggesting that approx. 2 Na⁺ ions participate in the transport of β -alanine, which is in good agreement with the data presented by Turner (1986). By contrast with the data for the high-affinity component, the initial uptake of β -alanine at 3 mM may be the result of contributions from both the high-affinity and low-affinity transport systems. Therefore we have subtracted the activity of the high-affinity component from the total Na⁺-dependent transport activity. The corrected values for the uptake of β -alanine (Fig. 1a) exhibit a simple hyperbolic curve as the Na⁺ concentration was increased (a Michaelis–Menten-type dependence). Fig. 1(b) illustrates that an n value of 1 provides the best fit to these data, since this value yields a straight line.

We also examined the coupling ratio of the H⁺- β -alanine co-transport system by measuring the initial uptake of β -alanine in the presence of increasing magnitudes of H⁺ gradients (extravesicular > intravesicular). The results are shown in Fig. 2, where all uptake values are corrected for the uptake in the absence of an H⁺ gradient (pH_{out} = pH_{in} = 7.5). As seen from the plot, $n = 1$ provides a straight-line relationship between flux/[H⁺] ^{n} against flux, indicating the involvement of 1 H⁺ per β -alanine-transport event in these vesicle preparations.

In order to investigate further the characteristics of the β -alanine-carrier complex, the stoichiometry of the Na⁺- β -alanine co-transport mechanism for Cl⁻ was studied. The initial uptake of β -alanine (700 nM) increased as the concentrations of Cl⁻ increased, and the shape of the curve was hyperbolic, revealing a Michaelis–Menten-type dependence. Thus these data suggests a 1 Cl⁻:1 β -alanine coupling ratio (results not shown).

The 'activation method' does not provide information as to

Fig. 3. Effect of Na⁺ concentration on the uptake of β -alanine by luminal membrane vesicles from pars recta

The preparation of vesicles and the composition of the incubation media were the same as described in the legend to Fig. 1. The uptake was studied at two different concentrations of β -alanine. All uptake values were corrected for the uptake in the absence of a Na⁺ gradient. (a) Plot of flux versus Na⁺ concentration for 600 nM- β -[3-³H(n)]alanine (uptake_{0 mM-Na⁺} = 1.39 pmol/min per mg of protein). (b) Plots of flux/[Na⁺] ^{n} versus flux for $n = 1$ (●) and $n = 2$ (○) for the high-affinity system (600 nM). The inset shows a series of plots of flux/[Na⁺] ^{n} against flux for different values of n ranging from 1.5 to 1.9. The units of [Na⁺] are M. (c) Plot of flux versus Na⁺ concentration for 750 nM- β -[3-³H(n)]alanine and unlabelled β -alanine reaching a final concentration of 3 mM (uptake_{0 mM-Na⁺} = 0.81 nmol/min per mg of protein). The curve shows the difference between flux at 3 mM and 600 nM. (d) Plots of flux/[Na⁺] ^{n} versus flux for $n = 1$ (●) and $n = 2$ (○) for the low-affinity system (3 mM minus 600 nM).

Fig. 4. Effect of Cl^- concentration on the uptake of β -alanine by luminal membrane vesicles from pars recta

Vesicles were preincubated in 20 mM-Hepes/Tris buffer, pH 7.5, containing 180 mM-potassium gluconate, 50 mM-sodium gluconate and valinomycin at a concentration of 12.5 μ g/mg of protein. The incubation media contained 50 mM-sodium gluconate, 700 nM- β -[3H](n)alanine and various concentrations of *N*-methyl-D-glucamine chloride ranging from 0 to 200 mM (final concn.). *N*-Methyl-D-glucamine sulphate replaced Cl^- iso-osmotically to obtain the various Cl^- concentrations studied. Uptake measured in the absence of Cl^- was subtracted from all points (uptake_{0 mM- Cl^-} = 2.40 pmol/min per mg of protein). The composition of the stop buffer was 20 mM-Hepes/Tris (pH 7.5)/200 mM-mannitol/300 mM-NaCl. (a) Plot of flux versus Cl^- concentration. (b) Plots of $flux/[Cl^-]^n$ versus flux for $n = 1$ ($\bullet$) and $n = 2$ ($\circ$). The units of $[Cl^-]$ are M.

whether the stimulation of β -alanine flux produced by the activator is the result of ion- β -alanine co-transport (energetic coupling) or of ion-induced facilitation of β -alanine entry without concomitant ion transport (catalytic coupling). However, our investigation of the stoichiometry of the Na^+ -dependent high-affinity transport system (2 Na^+ :1 Cl^- :1 β -alanine) confirms previous reports (Turner, 1986), which also concluded that β -alanine uptake involves the net transfer of positive charge and can be driven against a concentration gradient by both an Na^+ and a Cl^- gradient. Furthermore, since the Na^+ -dependent low-affinity and the H^+ -dependent transport systems are electrogenic processes, it is reasonable to suggest that these cations are co-transported with β -alanine in vesicles from pars convoluta.

Stoichiometry of Na^+ - and Cl^- -dependent β -alanine transport in vesicles from pars recta

The dependence of β -alanine uptake on Na^+ concentration is shown in Fig. 3. Initial flux rates of 600 nM- β -alanine and 3 mM- β -alanine were measured as a function of Na^{2+} concentration over the range 0–200 mM. At 600 nM- β -alanine (Fig. 3a), the sigmoidal shape of the plot of uptake against Na^+ concentration is indicative of the involvement of more than one Na^+ ion in the β -alanine transport process. An estimate of the number of Na^+ ions involved is found from the graphical plot in Fig. 3(b), indicating a 1.9:1 stoichiometry. In Fig. 3(c), the uptake values at 3 mM- β -alanine have been corrected by subtracting the uptake at 600 nM- β -alanine in order to isolate the contribution from the low-affinity transport system. These data have also been analysed as described under 'Calculations' in the Experimental section, and the plot in Fig. 3(d) shows a 1:1 stoichiometry for Na^+ - β -alanine co-transport by luminal membrane vesicles from pars recta of rabbit proximal tubule.

The stoichiometry of the Na^+ - β -alanine co-transport mechanism for Cl^- is given in Fig. 4. As in pars convoluta, the β -alanine uptake exhibited a hyperbolic dependence of flux on Cl^- concentration (Fig. 4a), indicating a 1 Cl^- :1 β -alanine coupling ratio (Fig. 4b).

In conclusion, the results presented here indicate that 1.8 Na^+ :1 Cl^- β -alanine (high-affinity), 1 Na^+ :1 β -alanine (low-affinity) and 1 H^+ :1 β -alanine are co-transported in vesicles from pars convoluta. In pars recta, 1.9 Na^+ :1 Cl^- (high-affinity) and 1 Na^+ (low-affinity) are involved in the transport of β -alanine.

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REFERENCES

- Hammerman, M. & Sacktor, B. (1978) *Biochim. Biophys. Acta* **509**, 338–347
- Hopfer, U., Nelson, K., Perrotto, J. & Isselbacher, K. J. (1973) *J. Biol. Chem.* **248**, 25–32
- Jessen, H., Jørgensen, K. E., Røigaard-Petersen, H. & Sheikh, M. I. (1989) *J. Physiol. (London)* **411**, 517–528
- Kragh-Hansen, U., Røigaard-Petersen, H. & Sheikh, M. I. (1985) *Am. J. Physiol.* **249**, F704–F712
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Sheikh, M. I. & Møller, J. V. (1987) in *Biochemical Toxicology: A Practical Approach* (Snell, K. & Mullock, B., eds.), pp. 153–182, IRL Press, Oxford
- Sheikh, M. I., Kragh-Hansen, U., Jørgensen, K. E. & Røigaard-Petersen, H. (1982) *Biochem. J.* **208**, 377–382
- Turner, R. J. (1986) *J. Biol. Chem.* **261**, 16060–16066
- Turner, R. J. & Moran, A. (1982a) *J. Membr. Biol.* **67**, 73–80
- Turner, R. J. & Moran, A. (1982b) *J. Membr. Biol.* **70**, 37–45
- Ullrich, K. J. (1979) *Annu. Rev. Physiol.* **41**, 181–195
- Wolff, N. A. & Kinne, R. (1988) *J. Membr. Biol.* **102**, 131–139