Renal transport of neutral amino acids

Cation-dependent uptake of L-alanine by luminal-membrane vesicles

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The characteristics of L-alanine transport in luminal-membrane vesicles isolated either from whole cortex or from pars convoluta or pars recta of rabbit proximal tubules were studied by a rapid filtration technique and by a spectrophotometric method. Uptake of L-alanine by vesicles from whole cortex was mediated by both Na⁺-dependent and Na⁺-independent, but electrogenic, processes. The nature, mechanism and tubular localization of the transport systems were studied by the use of vesicles derived from pars convoluta and pars recta. In vesicles from pars recta transport of L-alanine was strictly dependent on Na⁺ and occurred via a dual transport system, namely a high-affinity (half-saturation 0.14 mM) and a low-affinity system (half-saturation 9.6 mM). The cation-dependent but Na⁺-unspecific transport system for L-alanine was exclusively localized to the pars convoluta, which also contained an Na⁺-preferring system of intermediate affinity (half saturation 2.1 mM). A closer examination of the mechanism of transport of L-alanine in vesicles from pars convoluta revealed that an H⁺ gradient (extravesicular > intravesicular) can drive the transport of L-alanine into the vesicles both in the presence and in the absence of Na⁺. The physiological importance of various L-alanine transporters is briefly discussed.

INTRODUCTION

The existence of an Na⁺-dependent reabsorptive system for L-alanine in the brush border of proximal tubules is well documented from renal clearance measurements (Block & Hubbard, 1962), micropuncture (Silbernagl, 1981) and electrophysiological experiments (Samarzija & Frømter, 1982), as well as from transport studies on membrane vesicles derived from whole renal cortex (Fass et al., 1977). The reabsorptive system for L-alanine has been shown to be highly specific for neutral α-amino acids (Fass et al., 1977). However, more recent studies have in general suggested the presence of two or more transport systems for the reabsorption of neutral α-amino acids in luminal-membrane vesicles isolated from pars convoluta and pars recta of the proximal tubule (Kragh-Hansen et al., 1984; Kragh-Hansen & Sheikh, 1984; Røigaard-Petersen & Sheikh, 1984; Røigaard-Petersen et al., 1987). In the present investigation we have extended our original observations on amino acid transport to encompass the characteristics of L-alanine transport in these membrane vesicles. As the result of this investigation we obtained evidence for the existence of at least three different Na⁺-dependent systems and a unique H⁺-gradient-dependent transport system for L-alanine. Tubular localization studies showed that the H⁺-L-alanine co-transport system is exclusively confined to pars convoluta. The kinetic parameters of these various Na⁺-dependent and H⁺-dependent amino acid transport systems were further studied.

EXPERIMENTAL

Materials

Tris (Trizma) base, Tris hydrochloride, Heps, Mes, L-alanine, L-phenylalanine and D-glucose were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium and potassium salts of D-glucconate were purchased from Fluka A.G., Buchs, Switzerland. L-[U-¹⁴C]Alanine (sp. radioactivity 171 mCi/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. 3,3'-Diethyloxadicarbocyanine iodide was supplied by Eastman Kodak Co., Rochester, NY, U.S.A. These and all other reagents were of A.R. grade.

Preparation and enzymic characterization of renal luminal-membrane vesicles

Luminal-membrane vesicles were isolated from whole cortex, from pars convoluta ('outer cortex') and from pars recta ('outer medulla') of the proximal tubule of rabbit kidney according to the method already described (Kragh-Hansen et al., 1984, 1985) and mentioned here only briefly. Outer cortical tissue was obtained by taking slices ≤ 0.3 mm thick from the surface of the kidney containing pars convoluta. Strips of outer medulla tissue approx. 1 mm thick (representing predominantly pars recta) were dissected from the outer stripe of outer medulla. We always prepared luminal-membrane vesicles from outer cortical and outer medullary tissue from the same kidneys, and the two preparations were performed in parallel by using the Ca²⁺-precipitation procedure previously described by Sheikh et al. (1982). Unless otherwise stated the vesicles were suspended in a solution containing 310 mM-mannitol and 15 mM-Hepes/Tris buffer, pH 7.5. In a series of experiments luminal-membrane vesicles were prepared and suspended in a solution containing 310 mM-mannitol and 15 mM-Mes/Tris buffer, pH 5.5. 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

Abbreviation used: FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone.

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enzyme markers as previously described (Sheikh et al., 1982). The activity of alkaline phosphatase in the luminal-membrane vesicle fractions from pars convoluta and from pars recta was enriched as compared with the corresponding homogenates by factors of 9.4 ± 1.8-fold and 13.2 ± 2.8-fold (n = 8) respectively. Leucine amino-peptidase activity was enriched as compared with the corresponding homogenates by factors of 17.5 ± 2.7-fold for pars convoluta and 29.7 ± 3.6-fold for pars recta. Na⁺ + K⁺-stimulated ATPase activity was decreased to 0.12 ± 0.09-fold and 0.40 ± 0.19-fold of that of homogenate in the case of pars convoluta and pars recta membrane vesicles respectively. The amount of protein was determined by the method of Lowry et al. (1951) as modified by Peterson (1977), with serum albumin (Sigma Chemical Co.) as a standard. As judged by D-glucose-uptake studies no, or only a minor, cross-contamination of pars convoluta and pars recta membrane vesicles takes place (Krægh-Hansen et al., 1984, 1985). All solutions used in this study were sterilized before use. The possible bacterial contamination of membrane vesicle preparations was examined by incubating the samples of vesicles on blood/agar plates and by electron microscopy. No bacteria were found in these preparations.

**Uptake experiments**

Uptake of L-alanine by various vesicle preparations was examined by Millipore filtration (Hopfer et al., 1973) and by a spectrophotometric method with potential-sensitive carbocyanine dye as previously described (Krægh-Hansen et al., 1982a). In brief, the principle of the spectrophotometric method is as follows. A 1.2 ml portion of a buffered aqueous solution of the potential-sensitive dye 3,3′-diethyloxadecarbocyanine iodide, 1.2 ml of a buffered salt solution and 60 μl of membrane vesicle suspension was mixed in a 1 cm-path-length cuvette. The cuvette was placed in an Aminco DW-2a u.v.–visible spectrophotometer with a constant temperature in the sample compartment of 20 °C. The salt anions permeate into the vesicles faster than the salt cations, resulting in a slight reversible hyperpolarization of the membranes. The hyperpolarization was recorded on the spectrophotometer, and at its maximum a small volume of a stock solution of one of the organic solutes or buffer was added, under magnetic stirring, through a small opening in the top of the sample compartment. Details of the individual experiments are given in the legends to the Figures.

**Calculations**

The Michaelis–Menten kinetics of the uptake of various concentrations of L-alanine were analysed. Theoretical saturation curves were fitted to the experimental data by using a computer-analysed statistical iteration procedure (Jacobsen et al., 1982).

**RESULTS**

**Uptake of L-alanine by vesicles from whole renal cortex**

Fig. 1(a) depicts the pattern of uptake of L-alanine (curves 1 and 2) and L-phenylalanine (curves 3 and 4) by luminal-membrane vesicles from whole renal cortex. It is seen that addition of L-alanine to vesicle/dye suspension both in the presence of an Na⁺ gradient (curve 1) and in the presence of a K⁺ gradient (curve 2) resulted in absorbance changes (ΔA) indicative of depolarizing

![Fig. 1. Uptake of L-alanine by luminal-membrane vesicles from whole renal cortex](image-url)

(a) Uptake of 5 mM-L-alanine (curves 1 and 2) and 5 mM-L-phenylalanine (curves 3 and 4) as registered by the potential-sensitive dye 3,3′-diethyloxadecarbocyanine iodide. Common experimental conditions were as follows: protein concentration 0.25 mg/ml, pH 7.5, temperature 20 °C and dye concentration 15 μM. The intravesicular medium was 310 mM-mannitol, whereas the extravesicular medium was 155 mM-NaCl (curves 1 and 3) or 155 mM-KCl (curves 2 and 4). In both intravesicular and extravesicular media 15 mM-Hepes/Tris was used as buffer system. The break in the curves, at 0 min, indicates addition of solute. All the spectral curves were corrected for the effect of adding a small volume of 15 mM-Hepes/Tris buffer alone (the medium of the solute's stock solutions). The spectrophotometer was operated in the dual-wavelength mode with 580 nm and 610 nm (reference wavelength). (b) Uptake of increasing concentrations of L-alanine in the presence of a 155 mM-NaCl gradient (curve 1) or a 155 mM-KCl gradient (curve 2). In the inset the results are shown as Eadie–Hofstee plots. v represent the absorbance change at substrate concentration [S]. Results are given as means ± S.D. for four experiments.
Renal transport of L-alanine

Fig. 2. Uptake of L-alanine by luminal-membrane vesicles from pars convoluta

(a) Uptake of 5 mM-L-alanine in the presence of 155 mM-NaCl (curve 1), 103 mM-Na2SO4 (curve 2) or 155 mM-sodium D-gluconate (curve 3). (b) Uptake of 5 mM-L-alanine in the presence of 155 mM-KCl (curve 1), 103 mM-K2SO4 (curve 2) or 155 mM-potassium D-gluconate (curve 3). (c) Uptake of 5 mM-L-alanine (curve 1) and 5 mM-D-glucose (curve 2) in the presence of 310 mM-mannitol. Curve 3 shows the effect of addition of 10 mM-FCCP in the vesicle dye suspension before the addition of L-alanine. For further experimental details see the text and Fig. 1 legend.

event(s) (for details see Krarg-Hansen et al., 1982a,b), strongly suggesting the existence of both Na+-dependent and Na+-independent electrogenic transport systems for L-alanine. By contrast, the rate of uptake of L-phenylalanine is strictly Na+-dependent, i.e. no depolarization is discernible in the presence of a KCl gradient (curve 4).

Fig. 1(b) shows the absorbance changes induced by addition of increasing concentrations of L-alanine to dye/vesicle suspensions in the presence of an Na+ gradient (curve 1) and a K+ gradient (curve 2) respectively. The inset in Fig. 1(b) shows Eadie–Hofstee analysis of the experimental data. In contrast with previous observations (Fass et al., 1977), a curvilinear plot is obtained in the presence of an NaCl gradient, which indicates the presence of multiple Na+-dependent transport systems for L-alanine in vesicles from whole cortex. L-Alanine transport is maintained on replacing an NaCl gradient with a KCl gradient, but under these conditions a straight-line relationship is obtained for L-alanine transport, suggesting that the Na+-independent uptake of this amino acid occurs via a single transport system. It should be noted that Na+-independent uptake is not related specifically to the presence of a KCl gradient. Replacing KCl by either choline chloride or tetraethylammonium chloride produces essentially the same result (not shown).

In the following we examine the tubular localization of the various L-alanine transport systems by the use of luminal-membrane vesicles isolated from pars convoluta and from pars recta (Krarg-Hansen et al., 1984, 1985).

Characteristics of L-alanine transport by vesicles from pars convoluta

Figs. 2(a) and 2(b) show absorbance changes (ΔA) caused by the addition of L-alanine to luminal-membrane vesicles from pars convoluta in the presence of various sodium salt and potassium salt gradients respectively. Application of L-alanine to these vesicles depolarizes the membrane potential to various extents both with a sodium salt gradient and with different potassium salt gradients. Fig. 2(c) describes the rate of uptake of L-alanine in the absence of Na+ and K+ gradients. In these experiments the vesicles were prepared and equilibrated in a medium containing 310 mM-mannitol dissolved in 15 mM-Hepes/Tris buffer, pH 7.5, and 60 μM of the vesicle suspension (protein concentration 15 mg/ml) was added to 2.4 ml of the same solution, containing potential-sensitive carboxcyanine dye. Immediately thereafter 100 μl of either a stock solution of L-alanine (curve 1) or D-glucose (curve 2) was added to the vesicle/dye suspension, to give a final concentration in the cuvette of 5 mM of these organic compounds. It is seen that addition of L-alanine, but not of D-glucose, caused depolarization of luminal-membrane vesicles, indicating an electrogenic transport process for L-alanine uptake. These findings were confirmed by examining the effect of an H+ ionophore on the rate of uptake of L-alanine in this system. Curve 3 shows that addition of L-alanine to the dye/vesicle suspension in the presence of the H+ ionophore FCCP abolished the transient depolarization of the membrane vesicles, indicating that the Na+-independent electrogenic uptake of L-alanine may be driven by H+-alanine co-transport.

The nature and mechanism of the Na+-independent uptake of L-alanine by vesicles from pars convoluta were further examined by the Millipore filtration technique (Hopfer et al., 1973). In the presence of an inwardly directed Na+ gradient (curve 1 of Fig. 3a) the accumulation of the amino acid in the vesicles is markedly stimulated, reaching a maximal value after about 90 s of incubation. Thereafter the intravesicular concentration.
Fig. 3. Cation-dependent uptake of L-alanine by luminal-membrane vesicles from pars convoluta.

(a) A 20 μl portion of the vesicle suspension (15 mg/ml) was incubated at different time intervals in 100 μl of incubation mixture consisting of 155 mM-NaCl, 50 μM-L-[14C]alanine in 15 mM-Hepes/Tris buffer, pH 7.5 [curve 1 (■)], or in 15 mM-Mes/Tris buffer, pH 5.5 [curve 2 (●)]. Curve 3 (▲) shows the effect of Na+ gradient-dependent uptake of L-alanine at a low pH but in the absence of an H+ gradient (pH_{in} = pH_{out} = 5.5). In these experiments the vesicles were preincubated with 310 mM-mannitol and 10 μM-FCCP in 15 mM-Mes/Tris buffer, pH 5.5, for 30 min and then incubated in 155 mM-NaCl, 50 μM-L-[14C]alanine and 10 μM-FCCP in 15 mM-Mes/Tris buffer, pH 5.5, at different time intervals. (b) The experimental conditions were essentially the same as in (a) except that NaCl is replaced by KCl at pH 5.5 [curve 1 (●)] and pH 7.5 [curve 2 (■)]. Curve 3 (▲) shows the effect of Na+ gradient-independent uptake of L-alanine at a low pH but in the absence of an H+ gradient (pH_{in} = pH_{out} = 5.5). The composition of stop buffers as well as washing solutions was the same as the various incubation media, but without L-alanine, used in different groups of experiments. The results shown are from a representative experiment.

of L-alanine decreases, indicating net efflux during dissipation of the Na+ gradient. Curve 2 shows the effect accomplished by the imposition of a simultaneous H+ gradient (pH_{out} 5.5) and Na+ gradient (extravesicular > intravesicular) on the uptake of L-alanine by the preparation of these vesicles. It is seen that this resulted in an approx. 2-fold increase in the transient accumulation of L-alanine. Curve 3 depicts the Na+ gradient-dependent uptake of L-alanine at a low pH and in the absence of an H+ gradient (i.e. pH_{in} = pH_{out} = 5.5). In these experiments the vesicles were preincubated for 30 min in a medium containing 310 mM-mannitol, 15 mM-Mes/Tris buffer, pH 5.5, and 10 μM-FCCP. The vesicle preparation was then centrifuged and resuspended in an appropriate amount of the above-mentioned medium at a protein concentration of 15 mg/ml. A 20 μl portion was mixed with 100 μl of incubation medium containing 50 μM-L-[14C]alanine, 10 μM-FCCP and 155 mM-NaCl in 15 mM-Mes/Tris buffer, pH 5.5. This procedure was performed to ensure that the uptake of radioactive L-alanine is now measured only in the presence of an extravesicular Na+ gradient (FCCP was added to both the preincubation and the final incubation medium to keep the intravesicular pH constant at 5.5 during the whole incubation period). It is obvious from curve 3 that the additional uptake of L-alanine seen in the absence of a pH gradient (curve 2) is abolished under these experimental conditions. Thus in addition to Na+ the transport of L-alanine is influenced by an H+ gradient in luminal-membrane vesicles from the pars convoluta.

Curve 1 in Fig. 3(b) illustrates the renal uptake of radioactive L-alanine in the presence of H+ and K+ gradients (extravesicular > intravesicular) but in the absence of Na+. In these experiments NaCl was replaced by equimolar concentration of KCl in the incubation medium. The purpose of performing these experiments was to investigate whether an H+ gradient alone can energize the uptake of L-alanine in membrane vesicles from this segment of proximal tubule. It is apparent from Fig. 3(b) that an inwardly directed H+ gradient enhanced the renal accumulation of L-alanine, resulting in an ‘overshoot’ in the absence of Na+. No ‘overshoot’ was observed in the presence of a KCl gradient alone (curve 2), when the intravesicular pH was equal to extravesicular pH at 7.5. Curve 3 shows the Na+ independent uptake of L-alanine at a low pH, in the absence of an H+ gradient (i.e. pH_{in} = pH_{out} = 5.5). The experimental conditions were essentially the same as described for curve 3 in Fig. 3(a) except that NaCl was replaced by 155 mM-KCl. No ‘overshoot’ was observed under these experimental conditions. The results shown in curve 3 strongly suggest that the Na+ independent accumulation of L-alanine is exclusively driven by an H+ gradient in luminal-membrane vesicles from the pars convoluta.

Fig. 4(a) depicts the uptake of radioactive L-alanine (10 s uptake value) at increasing medium concentrations of the amino acid in the presence of an Na+ gradient alone (curve 1) and in the presence of both an Na+ gradient and an H+ gradient (curve 2). It appears from Fig. 4(a) that application of an H+ gradient in addition to an Na+ gradient resulted in a rapid and drastic increase in the uptake of L-alanine by these membrane vesicles. Curve 3 shows the uptake of L-alanine in the presence of a KCl gradient but in the absence of Na+ and H+ gradients. The uptake of L-alanine under these experimental conditions was proportional to medium concentration of amino acid and thus exhibited simple diffusion properties. Curve 4 illustrates the uptake of L-alanine in the presence of an H+ gradient. It appears from the curve that in the absence of Na+ the H+ gradient markedly stimulated the uptake of L-alanine in these
Renal transport of L-alanine

Vol. 248

Fig. 4. Cation-dependent uptake of L-alanine at increasing concentrations by luminal-membrane vesicles from pars convoluta

(a) A 20 μl portion of the vesicle suspension was added to 100 μl of incubation medium containing 155 mM-NaCl in 15 mM-Hepes/Tris buffer, pH 7.5 [curve 1 (■)], 155 mM-NaCl in 15 mM-Mes/Tris buffer, pH 5.5 [curve 2 (●)], 155 mM-KCl in 15 mM-Hepes/Tris buffer, pH 7.5 [curve 3 (▲)], or 155 mM-KCl in 15 mM-Mes/Tris buffer, pH 5.5 [curve 4 (□)]. The media contained 50 mM-L-[4C]alanine and various concentrations of unlabelled L-alanine ranging from 0.2 to 10 mM. (b) Effect of H+ gradient in the presence of Na+ [curve 1 (●)] and in the absence of Na+ [curve 2 (□)]. (c) Uptake of increasing concentrations of L-alanine in the presence of an NaCl gradient alone. In the inset the results are shown as an Eadie–Hofstee plot.

Fig. 5. Uptake of L-alanine by luminal-membrane vesicles from pars recta

(a) Uptake of 5 mM-L-alanine as registered by the spectrophotometric method. The intravesicular medium was 310 mM-mannitol and the extravesicular medium was 155 mM-NaCl (curve 1) or 155 mM-KCl (curve 2). (b) Uptake of increasing concentrations of L-alanine in the presence of a 155 mM-NaCl gradient. In the inset the results are shown as an Eadie–Hofstee plot. For further experimental details see Fig. 1 legend. Results are given as means ± s.d. for three experiments.

membrane vesicles. The effect of the H+ gradient as such is the same in the presence (i.e. curve 2 minus curve 1) and in the absence (i.e. curve 4 minus curve 3) of Na+ as shown in Fig. 4(b) with $K_m$ 4.4 mM and $V_{max}$ 5.1 nmol/10 s per mg of protein. In the presence of an NaCl gradient alone (i.e. curve 1 minus curve 3) the $K_m$ was 2.1 mM and $V_{max}$ 5.9 nmol/10 s per mg of protein as calculated from the data of Fig. 4(c).

Uptake of L-alanine by vesicles from pars recta

Fig. 5(a) shows the absorbance changes produced by the addition of L-alanine to luminal-membrane vesicles from pars recta in the presence of an NaCl gradient (curve 1) and a KCl gradient (curve 2) respectively. It is seen that application of L-alanine to vesicle/dye suspensions in the presence of an Na+ gradient depolarizes the membrane potential. By contrast, no depolarization was observed when NaCl was replaced by KCl, indicating that the uptake of the amino acid by these vesicle preparations is strictly Na+-dependent. Fig. 5(b) depicts the optical response induced by increasing concentrations
of L-alanine to the vesicle/dye suspension in the presence of an Na⁺ gradient. The inset shows the Eadie–Hofstee analysis of the experimental data. A curvilinear plot is obtained, which suggests the presence of more than one Na⁺-dependent transport system for L-alanine uptake by vesicles from pars recta. We have assumed the presence of two different transport systems, and our computerized calculations (Jacobsen et al., 1982) showed that the experimental results are in accordance with such a model. The values for $K_{v,1}$ and $K_{v,2}$ were found to be 0.14 mm and 9.6 mm respectively.

**DISCUSSION**

In contrast with previous observations (Fass et al., 1977), the results reported in the present paper clearly show the presence of multiple transport systems for the renal reabsorption of L-alanine. At least two strictly Na⁺-dependent electrogenic systems for the transport of L-alanine exist in pars recta, with high affinity and low affinity. By contrast, in membrane vesicles from pars convoluta the uptake of L-alanine occurred by both Na⁺-dependent and Na⁺-independent electrogenic transport processes. The results shown in Figs. 3(a) and 3(b) provide direct evidence for coupling between L-alanine transport and H⁺ fluxes across the luminal-membrane vesicles obtained from pars convoluta. The strongest evidence supporting H⁺-L-alanine co-transport is the ability of a pH gradient (alkaline inside) to drive net L-alanine accumulation in the absence of other energy sources [curve I in Fig. 3(b)]. The results of kinetic experiments showed that the stimulation of L-alanine uptake by an H⁺ gradient was additive to that produced by Na⁺. These observations thus strongly suggest the existence of both Na⁺-dependent and H⁺-dependent transporters in luminal-membrane vesicles isolated from pars convoluta of rabbit proximal tubule. In this connection, it may be noted that studies on the mechanisms of renal transport of L-proline showed the presence of H⁺-L-proline co-transporter in luminal-membrane vesicles isolated from pars convoluta (Røigaard-Petersen et al., 1987). Whether H⁺-L-proline co-transporter can accommodate L-alanine as a transport substrate, however, remains to be investigated.

It is already known that the uptake of various amino acids across bacterial membrane is driven by a pH gradient (Plate & Suit, 1981). However, until now the stimulation of the L-alanine transport by a pH gradient has not been reported in luminal-membrane vesicles from a mammalian kidney proximal tubule. We have previously reported that an Na⁺/H⁺ antipporter predominantly functions in pars convoluta of rabbit proximal tubule (Krøgh-Hansen et al., 1985; Jacobsen et al., 1986). We conclude that the coexistence of Na⁺/H⁺ exchange and H⁺-sensitive alanine uptake by luminal-membrane vesicles from pars convoluta may have physiological significance for the mechanism for renal reabsorption of L-alanine in vivo in this region of proximal tubule.

The existence of a high-affinity and a low-affinity transport system for L-alanine in vesicles from pars recta is not an unexpected finding, since we previously obtained similar results on the mechanism of uptake of other neutral α-amino acids, namely L-phenylalanine (Krøgh-Hansen et al., 1984) and L-serine (Krøgh-Hansen & Sheikh, 1984) by luminal-membrane vesicles from pars recta. The high-affinity system for L-alanine transport localized in pars recta might be used for the efficient reabsorption of residual amounts of L-alanine present in this region of the nephron (for a detailed discussion of this aspect of amino acid reabsorption see Krøgh-Hansen & Sheikh, 1984).

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