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Mechanism of transport of L-alanine by luminal-membrane vesicles from pars recta of rabbit proximal tubule

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The characteristics of renal transport of L-alanine by luminal-membrane vesicles from proximal straight tubules (pars recta) of rabbit kidney were investigated. The following picture emerges from transport studies. Two electrogenic and Na^+ requiring systems confined to this region of the nephron exist for the transport of L-alanine. In addition to Na^+, the transport of L-alanine was influenced by H^+. However, H^+ does not substitute for Na^+, but instead potentiates the Na^+ effect. Modification of histidyl residues of the intact luminal-membrane vesicles by diethylpyrocarbonate (DEP), completely abolished the transient renal accumulation of L-alanine. Substrate and Na^+-protection experiments suggest that histidyl residues may be at or close to the active site of the L-alanine transporter in membrane vesicles from pars recta.

Transport of L-alanine; Na^+ dependence; pH dependence; Chemical modification; Pars recta; (Rabbit kidney)

1. INTRODUCTION

Recently, we have provided evidence for the existence of at least three different Na^+-dependent systems and a unique H^+ gradient dependent transport system for the renal reabsorption of L-alanine [1]. The nature, mechanism and tubular localization of these systems have been studied by the use of luminal-membrane vesicles prepared from pars convoluta and pars recta. In pars convoluta of the rabbit proximal tubule two transport systems have been characterized: (1) a Na^+-dependent system with intermediate affinity (half-saturation: 2.1 mM), and (2) a Na^+-independent system, which in the presence of a H^+ gradient (extravesicular > intravesicular) can drive the transport of L-alanine into these vesicles. In vesicles from pars recta transport of L-alanine was strictly dependent on Na^+ and mediated by a dual transport system, namely a high affinity system (half-saturation: 0.14 mM) and a low affinity system (half-saturation: 9.6 mM). We have subsequently found that the systems located in pars recta are also stimulated by a H^+ gradient. However, H^+ does not substitute for Na^+, as it apparently does in pars convoluta, but instead potentiates the Na^+ effect. We are thus confronted with a different phenomenon than previously for the H^+-sensitive transport of L-alanine in pars convoluta. This aspect of the Na^+-dependent cotransport systems in pars recta is the subject of this communication.

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2. MATERIALS AND METHODS

2.1. *Preparation of luminal-membrane vesicles from pars recta*

Luminal-membrane vesicles were isolated from pars recta ('outer medulla') of the proximal tubule of rabbit kidney according to [2,3] and the method is only briefly described here. Strips of outer medulla tissue approx. 1 mm (representing predominantly pars recta) were dissected from the outer strip of outer medulla. The renal tissue was homogenized and luminal-membrane vesicles were prepared by differential centrifugation and Ca^{2+} precipitation as in [4]. 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 regarding the content of luminal vesicles was examined by electron microscopy [3] and by measuring specific activities of various enzyme markers as in [4]. The amount of protein was determined as in [5] as modified in [6] with serum albumin (Sigma, St. Louis, MO, USA) as a standard. All solu-

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tions used in this study were sterilized before use. The possible bacterial contamination of membrane vesicle preparations was examined by incubating the samples on blood-agar plates and by electron microscopy. No bacteria were found in these preparations.

2.2. *Modification of histidyl residues*

Diethylpyrocarbonate (DEP) is known to react with the imidazole group of histidyl residues [7]. Recently, Bindslev and Wright [8] have reported that DEP can be successfully used to modify histidyl residues of the intact luminal-membrane vesicle proteins prepared from whole kidney cortex. Exactly the same procedures have been used in this study, to modify the histidyl residues of the luminal-membrane vesicles isolated from the pars recta of rabbit proximal tubule.

2.3. *Uptake of L-alanine by membrane vesicles*

The uptake of L-alanine by luminal-membrane vesicles was studied by Millipore filtration [9]. The details of the individual experiments are given in the legends to the figures.

3. RESULTS AND DISCUSSION

The time course for the uptake of radioactive L-alanine by luminal-membrane vesicles isolated from pars recta of rabbit proximal tubule suspended in various incubation media was measured by Millipore filtration. The results are summarized in fig.1. The presence of sodium salt gradient between the external medium and the intravesicular medium (curve 1) stimulated uptake of L-alanine in the membrane vesicles. The transient renal accumulation of L-alanine was maximal at about 90 s. Afterwards the amount of amino acid in the vesicles decreased, indicating net efflux of this compound. Curve 2 shows the effect accomplished by H^+ and Na^+ gradient (extravesicular > intravesicular) on the uptake of L-alanine by these vesicle preparations. It is apparent from the experimental data plotted in curve 2 that the imposition of H^+ gradient resulted in an approx. two-fold increase in the Na^+-dependent transient accumulation of L-alanine. Curves 3 and 4 show the uptake of L-alanine in the presence of K^+ and H^+ plus K^+ gradients, respectively. No 'overshoot' was observed in the presence of K^+ or H^+ plus K^+ gradient. The results of these experiments clearly demonstrate that the transient uptake of L-alanine in membrane vesicles from this segment of proximal tubule is strictly dependent on the Na^+ gradient. These findings are in contrast to the results obtained on the mechanism of L-alanine transport by membrane vesicles isolated from the pars convoluta of rabbit proximal tubule (for comparison

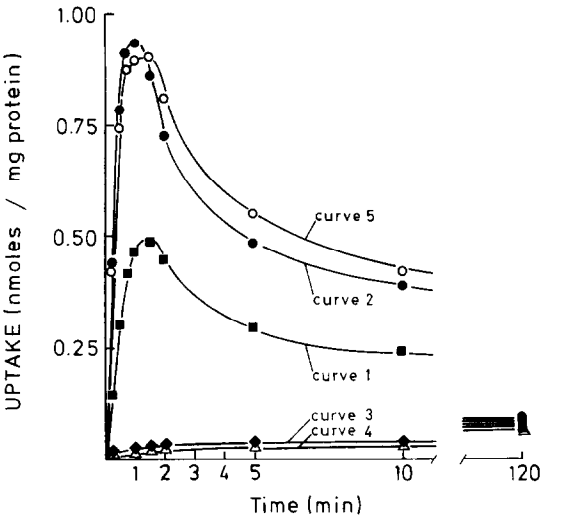

Fig.1. Cation-dependent uptake of L-alanine. 20 μl of vesicle suspension (15 mg/ml), prepared in 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, were incubated at different time intervals in 100 μl incubation mixture consisting of 155 mM NaCl, 50 μM L-[^{14}C]alanine in 15 mM Hepes/Tris, pH 7.5 (curve 1, ■) or in 15 mM Mes/Tris, pH 5.5 (curve 2, ●). Curves 3 and 4 show the effect of substituting NaCl by KCl in 15 mM Hepes/Tris, pH 7.5 (◆) or in 15 mM Mes/Tris, pH 5.5 (△), respectively. Curve 5 (○) shows the time course of incubating 20 μl of vesicle suspension (15 mg/ml), prepared in 310 mM mannitol, 15 mM Mes/Tris, pH 5.5, in 100 μl incubation mixture consisting of 155 mM NaCl, 50 μM L-[^{14}C]alanine, 10 μM FCCP in 15 mM Mes/Tris, pH 5.5. Composition of stop-buffers as well as washing solutions was the same as various incubation media without L-alanine used in different groups of experiments.

see curve 1 of fig.3b in [1]). To examine whether the Na^+-dependent transient accumulation of L-alanine in vesicles from pars recta can be achieved by a lower pH without pH gradient (i.e. $pH_{in} = pH_{out} = 5.5$), a series of experiments were performed. In these experiments luminal-membrane vesicles from pars recta were prepared in medium containing 310 mM mannitol in 15 mM Mes/Tris buffer (pH 5.5). These vesicles were then incubated in a medium containing radioactive L-alanine and 155 mM NaCl dissolved in 15 mM Mes/Tris buffer, pH 5.5 (curve 5 of fig.1). This procedure was performed to ensure that the uptake of L-alanine is measured only in the presence of an extravesicular Na^+ gradient. The comparison between the results plotted in curve 5 (lower pH without pH gradient) and curve 2 (pH gradient) revealed that the magnitude of transient accumulation of L-alanine is not significantly different

under these experimental conditions. These findings are in contrast to the results obtained on the mechanism of L-alanine transport by vesicles from the pars convoluta (for details see curves 2 and 3 of fig.3a in [1]). These results thus strongly indicate that the lower pH but not the pH gradient itself is responsible for the stimulation of Na^+ gradient dependent uptake of L-alanine in membrane vesicles from pars recta.

Fig.2 depicts the uptake of radioactive L-alanine (10 s uptake values) at increasing medium concentrations of the amino acid in the presence of a Na^+ gradient alone (curve 1) and in the presence of both Na^+ and H^+ gradient (curve 2). The values given in curves 1 and 2 have been corrected for passive diffusion by subtracting the uptake values obtained in the presence of an equimolar concentration gradient of KCl. It appears from the figure that application of H^+ gradient in addition to Na^+ resulted in a rapid and drastic increase in the uptake of L-alanine by these membrane vesicles. The inset shows the Eadie-Hofstee analysis of the experimental data. Curvilinear plots were obtained which confirm the existence of multiple transport systems previously reported [1] for the uptake of L-alanine in luminal-membrane vesicles from the pars recta. We have assumed the presence of two different transport systems, and our computerized calculations showed that the experimental results are in accordance with such a model. K_{m1} and K_{m2} values for both the Na^+-dependent and Na^+- plus H^+-dependent transport systems were found to be the same, whereas V_{max1} and V_{max2} values for these systems were different and were calculated to be as follows: Na^+-dependent systems, K_{m1} = 0.25 mM, K_{m2} = 9.0 mM, V_{max1} = 2.0 nmol/mg protein/10 s, V_{max2} = 5.1 nmol/mg protein/10 s; Na^+- plus H^+-dependent systems, K_{m1} = 0.23 mM, K_{m2} = 9.7 mM, V_{max1} = 5.7 nmol/mg protein/10 s, V_{max2} = 19.5 nmol/mg protein/10 s.

The results presented above suggest that the Na^+-dependent stimulation of L-alanine uptake induced by pH changes may be due to the protonation of specific membrane group(s). We have attempted to investigate the possible role of histidyl residues of the membrane protein(s) involved in the transport of L-alanine.

Curve 1 in fig.3a describes the uptake of radioactive L-alanine by non-DEP-treated luminal-membrane vesicles (control) in the

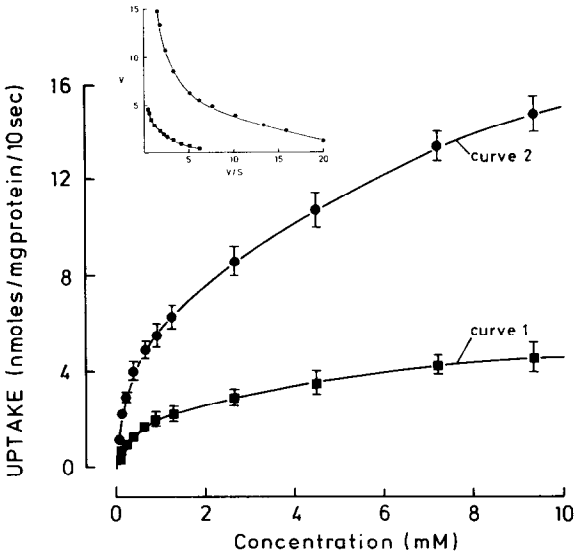

Fig.2. Kinetics of L-alanine uptake. (a) 20 μl of vesicle suspension (15 mg/ml), prepared in 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5, were added to 100 μl incubation medium containing 155 mM NaCl in 15 mM Hepes/Tris, pH 7.5 (curve 1, ■) or 155 mM NaCl in 15 mM Mes/Tris, pH 5.5 (curve 2, ●). The media contained 50 μM L-[^{14}C]alanine and various concentrations of unlabelled L-alanine ranging from 0–10 mM (final concentration). In the inset the results are shown in Eadie-Hofstee plots. Results are given as mean values ± SD of three experiments.

presence of Na^+ plus H^+ gradient. Curve 2 shows the corresponding uptake of L-alanine by DEP-treated membrane vesicles in the presence of Na^+ plus H^+ gradient. It can be seen from the figure that transient accumulation of L-alanine is completely abolished in DEP-treated membrane vesicles under these experimental conditions. In order to establish that DEP-treatment specifically inhibits L-alanine transporter located in these vesicle preparations, substrate and Na^+-protection experiments as in [8] were performed. It is apparent from fig.3b that only L-alanine in the DEP-reaction medium gave protection of L-alanine transport. Thus with 10 mM L-alanine and 130 mM NaCl in the reaction medium, the L-alanine transporter was protected 60 ± 5%. Furthermore, pretreatment of vesicles with DEP reduces the Na^+-dependent uptake of L-lactate and D-glucose to 70 ± 5% and 6 ± 3% of the control values, respectively. L-alanine at 10 mM medium concentration does not protect either L-lactate or D-glucose transport, nor does 10 mM L-lactate or 10 mM D-glucose in the presence of

130 mM NaCl protect any of the transporters (not shown). Furthermore, Na^+ in the reaction medium is an obligatory requirement for the protection of the L-alanine transporter from DEP attack. Replacing all Na^+ with either K^+ or choline ions prevented the protective effect of L-alanine (not shown). These results strongly suggest the involvement of histidyl residues in the active transport of L-alanine across the luminal membrane of renal cells from pars recta.

In conclusion, the results presented here show that the Na^+-dependent uptake of L-alanine in luminal-membrane vesicles from pars recta is stimulated at lower pH. Thus in addition to Na^+ the transport of L-alanine is influenced by H^+. However, H^+ does not substitute for Na^+, but instead potentiates the Na^+ effect. An attempt to find the residues most likely situated in the substrate and/or Na^+ sites revealed that a histidyl residue may be at or close to the active site of the L-alanine transporter in vesicles from pars recta.

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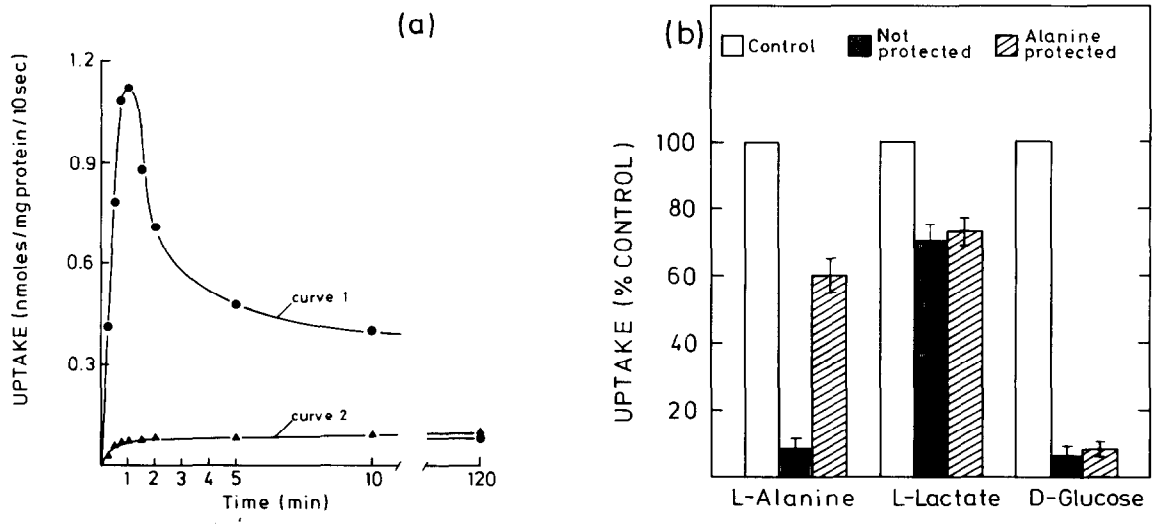

Fig.3. Diethylpyrocarbonate effect on L-alanine uptake. (a) Membrane vesicles were equilibrated in 310 mM mannitol, 15 mM Hepes/Tris, pH 6.4 for 30 min. DEP was diluted 1:1 (v/v) in ethanol and this solution was further diluted 1:50 (v/v) by the above-mentioned buffer. This DEP solution was added to the vesicle preparation until a DEP concentration of 5 mM (final concentration) and left under continuous stirring at 20°C for 5 min. The reaction was stopped by adding icecold buffer (310 mM mannitol, 15 mM Hepes/Tris, pH 7.5). The solution was centrifuged and the pellet dissolved in 310 mM mannitol, 15 mM Hepes/Tris, pH 7.5. This step was repeated three times. 20 μl DEP-treated vesicle suspension (15 mg/ml) was incubated at different time intervals in 100 μl incubation medium consisting of 155 mM NaCl, 50 μM L-[^{14}C]alanine in 15 mM Mes/Tris, pH 5.5 (curve 2, ▲). As control was used untreated vesicle suspension subjected to the same experimental procedure (curve 1, ●). (b) A vesicle preparation was divided into three parts: (1) one was left as control but treated as the other parts, (2) one was treated with DEP as described above, and (3) one was DEP-modified under similar conditions but in the presence of 10 mM L-alanine and 130 mM NaCl in the reaction solution. Uptake was measured by adding 20 μl of one of the above-mentioned vesicle suspensions to 100 μl incubation medium containing 155 mM NaCl, 50 μM L-[^{14}C]alanine or L-[^{14}C]lactate or D-[^{14}C]glucose in 15 mM Hepes/Tris, pH 7.5 for 30 s. Results are given as mean values ± SD of three experiments.