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Electrogenic uptake of D-imino acids by luminal membrane vesicles from rabbit kidney proximal tubule

Hans Røigaard-Petersen, Christian Jacobsen, Henrik Jessen, Steen Mollerup and M. Iqbal Sheikh

Institute of Medical Biochemistry, University of Aarhus, Aarhus (Denmark)

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Some characteristics of electrogenic uptake of D-proline and hydroxy-D-proline by luminal membrane vesicles isolated either from pars convoluta or from pars recta of rabbit proximal tubule were indirectly studied by the spectrophotometric method. In vesicles from pars convoluta, the uptake of D-imino acids was mediated by both Na^+-dependent and Na^+-independent, but electrogenic processes. Indirect evidence for coupling between D-imino acids and H^+ fluxes was obtained by the following observations: (1) Addition of the H^+ ionophore (FCCP) to the vesicle-dye (3,3'-diethyloxadicarbocyanine iodide) suspension completely abolished the Na^+-indedpendent electrogenic uptake of D-proline and hydroxy-D-proline by membrane vesicles from pars convoluta. (2) Addition of a relatively low concentration of D-proline in the incubation system decreased the H^+-gradient dependent renal uptake of radioactive L-proline to approx. 60% of the control value. By contrast, the uptake of D-proline in vesicles from pars recta was strictly Na^+-dependent, since no transient depolarization of membrane vesicles was ever observed in the absence of Na^+. A comparison between the transport characteristics of D-imino acids and their naturally occurring L-isomers indicated that these compounds probably share common transport systems located along the proximal tubule of rabbit kidney.

Introduction

In previous papers we have reported in detail the chracteristics of uptake of L-proline by luminal membrane vesicles isolated from different segments of rabbit proximal tubule [1–3]. Transport of L-proline was shown to be mediated by both an Na^+-dependent and an Na^+-independent system, which in the presence of inwardly directed H^+ gradient can drive the uphill transport of this compound into these vesicles [2,3]. However, no conclusive information exists in the literature on the mechanism of transport or the segmental localization of D-isomers of these imino acid reabsorption sites along the proximal tubule of mammalian kidney. The purpose of this investigation is to examine (1) the role of various cations on the rate of uptake of D-proline and hydroxy-D-proline and (the tubular localization of D-imino acid transport, and finally (3) to investigate whether D- and L-isomers of these imino acids share common transport system(s).

Methods

Preparation of renal luminal membrane vesicles

Luminal membrane vesicles were isolated from either pars convoluta ('outer cortex') or pars recta ('outer medulla') of the proximal tubule of rabbit kidney according to the method previously described [4] 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 Ca^{2+} precipitation procedure previously described [4]. Unless otherwise stated the vesicles were suspended in a solution containing 310 mm mannitol and 15 mm Hepes-Tris buffer (pH 7.5). The purity of the membrane vesicle preparation with regard to the content of luminal vesicles was examined by electron microscopy and by measuring specific activities of various enzyme markers as previously described [5,6]. The amount of

Correspondence: M.I. Sheikh, Institute of Medical Biochemistry, University of Aarhus, DK-8000 Aarhus C, Denmark.

protein was determined by the method of Lowry et al. [7] and as modified by Peterson [8], with serum albumin (Sigma Chemical Co.) as a standard. 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 D-proline and hydroxy-D-proline by various vesicle preparations was examined by a spectrophotometric method with potential-sensitive carbocyanine dye as previously described [9]. 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′-diethyloxadicarbocyanine iodide, 1.2 ml of a buffered salt solution and 60 μl of membrane vesicle suspension were mixed in a 1 cm path-length cuvette. The cuvette was placed in an Aminco DW-2a ultraviolet-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.

In a series of experiments, the effect of D-proline on the Na^+- and H^+-gradient dependent uptake of radioactive L-proline in vesicles from pars convoluta and pars recta of rabbit proximal tubule was examined by the use of Millipore filtration technique [10]. Briefly, the uptake of radioactive L-proline in the absence and presence of D-proline was studied as follows. 20 μl of a luminal membrane vesicle suspension were added at time 0 to 100 μl of incubation medium containing 310 mM mannitol, or 155 mM NaCl dissolved in either 15 mM Hepes-Tris buffer (pH = 7.5) or 15 mM Mes-Tris (pH = 5.5). Transport of L-proline in vesicles was stopped by addition of 1 ml ice-cold stop buffer. The resulting suspension was rapidly filtered through a Sartorius membrane filter (0.45 μm, type SM 11106, Grötting, F.R.G.) that was washed twice with 2.5 ml of ice-cold stop buffer. The filter was dried overnight and the radioactivity was counted in a liquid scintillation counter (Wallac LKB 1210 Ultra-beta) in Luma gel (Lumac, The Netherlands). Correction for nonspecific binding to the filter and membrane vesicles was made by subtracting from all uptake data the value of blank obtained by filtering denatured membranes (boiled for 2 min) added to an incubation tube containing radioactive L-proline.

Calculations

The results of the saturation experiments were analyzed according to the following equation

$$\Delta A = \frac{\Delta A_{max}[S]}{K_A + [S]}$$

where ΔA is the absorbance measured by addition of D-imino acids at concentration [S], ΔA_{max} is the maximal absorbance change and K_A is the D-imino acid concentration producing a half-maximal absorbance. The kinetic parameters were calculated using iteration computer programs [11].

Results

Uptake of D-proline and hydroxy-D-proline by luminal membrane vesicles from pars convoluta

Figs. 1 and 2 record ΔA caused by the addition of D-imino acids by luminal membrane vesicles from pars convoluta in the presence of various sodium salts and potassium salt gradients, respectively. Application of D-proline and hydroxy-D-proline to these vesicles depolarizes the membrane potential to various extents both with sodium salt gradients and with different potassium salt gradients.

Figs. 3A and 3B show ΔA produced by the addition of D-imino acids at increasing medium concentrations in the presence of NaCl and KCl gradient, respectively. Insets in the figures depict Eadie-Hofstee analysis of the data. Plots with straight line relationship were obtained for both the Na^+-dependent and K^+-dependent uptake of D-proline and hydroxy-D-proline, suggesting that the uptake of these compounds occurred via a single, low-affinity transport system (see Table I).

The nature and mechanism of the Na^+-independent uptake of D-imino acids by vesicles from pars convoluta was examined as follows: In a series of 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 μl of the vesicle suspension (protein concentration 15 mg/ml) was added to 2.4 ml of the same solution, containing potential-sensitive carbocyanine dye. Afterwards, 100 μl of either a stock solution of D-imino acids (curves 1 and 2 of Fig. 4) or D-glucose (curve 3 of Fig. 4) were added to the vesicle-dye suspension, to give a final concentration in the cuvette of 5 mM of these organic compounds. It is apparent from Fig. 4 that addition of D-proline and hydroxy-D-proline, but not of D-glucose, caused depolarization of luminal membrane vesicles, indicating a Na^+-independent electrogenic transport process for D-imino acid uptake. In mannitol buffer medium in the absence of a cation gradient the depolarization observed is caused by the trans D-imino acid chemical gradient (i.e., extravesicular D-imino acid

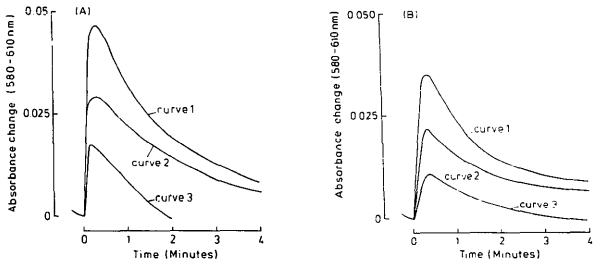

Fig. 1. Rate of uptake of D-proline (A) and hydroxy-D-proline (B) by luminal membrane vesicles from pars convoluta in the presence of an Na^+ gradient. Curves 1, 2, and 3 show uptake with NaCl, Na_2SO_4 or sodium gluconate as extravesicular medium, respectively. The final concentration of the D-imino acids was 5 mM. The protein concentration was 0.91 mg/ml. The dye concentration was 15 μM. Intravesicular pH was the same as extravesicular pH (i.e., $pH_{in} = pH_{out}$). The break in the curves, at time zero, indicates the addition of solutes. All the curves were corrected for the effect of adding a small volume of 15 mM Tris-HCl buffer alone (the medium of the stock solutions of the solute). The spectrophotometer was operated in the dual wavelength mode with 580 nm and 610 nm (reference wavelength).

concentration = 5 mM and intravesicular D-imino acid concentration = 0 at the start of the experiment). Since D-imino acids are neutral molecules, they must cotransport with some cation other than Na^+ or K^+, otherwise there should not be a depolarization of membrane vesicles after addition of the D-imino acids to the vesicle dye suspension. To investigate, whether the cation cotransported with neutral D-imino acid is H^+, the effect of a H^+ ionophore (FCCP) on the rate of uptake of D-imino acids is examined. Curve 4 shows that addition of D-proline and hydroxy-D-proline to the vesicle suspension in the presence of FCCP abolished the transient depolarization of the membrane vesicles, indicating that the Na^+-independent electrogenic uptake of D-imino acids may be driven by H^+/D-imino acid cotransport. The direct coupling between D-imino acids and H^+ fluxes cannot be explored by the spectrophotometric method, since both the ΔA_{max} and the spectral curve of the dye are dependent on pH.

Uptake of D-proline and hydroxy-D-proline by luminal membrane vesicles from pars recta

Fig. 5 shows ΔA induced by addition of D-proline and hydroxy-D-proline to membrane vesicles from pars recta in the presence of various sodium salts or a KCl gradient. It is seen that only D-proline depolarizes the membrane vesicles in the presence of an NaCl gradient. No depolarization was observed after application of hydroxy-D-proline to the vesicle dye suspension in the presence and absence of a NaCl gradient. Furthermore, when sodium salt was replaced by KCl or mannitol, no 'overshoot' was observed after addition of D-proline, indicating that the electrogenic uptake of D-proline by these vesicle preparations is strictly Na^+-dependent.

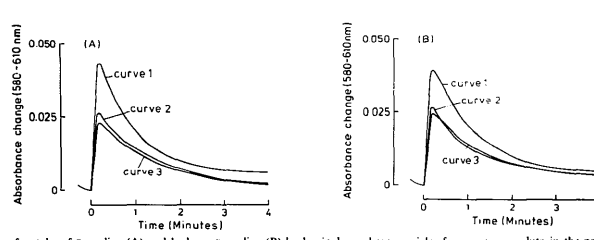

Fig. 2. Rate of uptake of D-proline (A) and hydroxy-D-proline (B) by luminal membrane vesicles from pars convoluta in the presence of a K^+ gradient. Curves 1, 2, and 3 show uptake with KCl, K_2SO_4 or potassium gluconate as extravesicular medium, respectively. For further details see legend to Fig. 1.

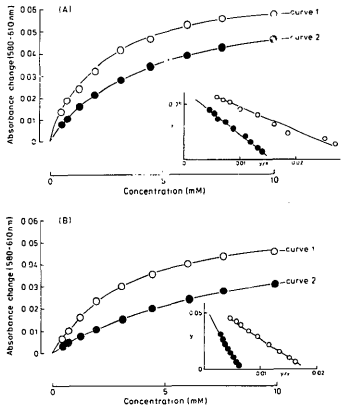

Fig. 3. (A) Kinetics of uptake of D-proline (curve 1) and hydroxy-D-proline (curve 2) by luminal membrane vesicles from pars convoluta in the presence of a Na^+ gradient. (B) Kinetics of renal uptake of D-proline (curve 1) and hydroxy-D-proline (curve 2) in the presence of a K^+ gradient. The inset in the figures show the Eadie-Hofstee analysis of the experimental data. For further details see legend to Fig. 1.

Fig. 6 records the optical response induced by increasing concentrations of D-proline in the presence of a Na^+ gradient. The rate of uptake of this compound is rapid at low concentrations and approaches saturation at higher medium concentrations. The inset in Fig. 6 depicts the Eadie-Hofstee analysis of the experimental data, showing that the uptake of D-proline occurs via a single transport system. The K_A value calculated is shown in Table I, together with those obtained for pars convoluta.

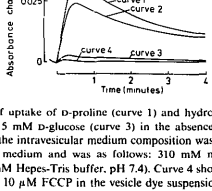

Fig. 4. Rate of uptake of D-proline (curve 1) and hydroxy-D-proline (curve 2) and 5 mM D-glucose (curve 3) in the absence of a cation gradient (i.e., the intravesicular medium composition was the same as extravesicular medium and was as follows: 310 mM mannitol dissolved in 15 mM Hepes-Tris buffer, pH 7.4). Curve 4 shows the effect of addition of 10 μM FCCP in the vesicle dye suspension before the addition of 5 mM D-proline or 5 mM hydroxy-D-proline. For further details see legend to Fig. 1.

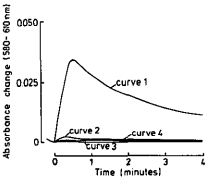

Fig. 5. Rate of uptake of D-proline (curves 1–3) and hydroxy-D-proline (curve 4) by luminal membrane vesicles from pars recta. Curves 1 and 2 show D-proline uptake in the presence of NaCl and Na_2SO_4 gradients, respectively. Curve 3 illustrates the uptake of D-proline in the presence of a KCl gradient. Curve 4 records the uptake of hydroxy-D-proline in the presence of NaCl gradient. For further details see legend to Fig. 1.

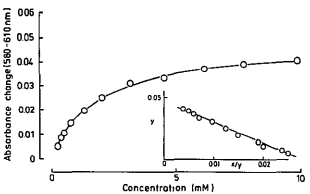

Fig. 6. Kinetics of uptake of D-proline by luminal membrane vesicles from pars recta in the presence of the NaCl gradient. The inset in the figure shows the Eadie-Hofstee analysis of the experimental data. For further details see legend to Fig. 1.

TABLE I

Affinities of various transport systems of pars convoluta and pars recta for D- and L-isomers of imino acids

The values given (average of three experiments) in the table are the half-saturation (K_A and K_m) values (in mM) for D- and L-imino acids.

Amino acid	Class	Pars convoluta		Pars rect	
		NaCl	KCl	NaCl	KCl
D-Proline	K_A	2.1	3.9	1.7	–
Hydroxy-D-proline	K_A	3.2	9.7	–	–
L-Proline	K_A^1	0.75	–	0.16	–
	K_A^2	9	4.5	–	–
	K_m^1	0.57	–	0.16	–
	K_m^2	9.8	–	–	–
Hydroxy-L-proline	K_A^1	1.3	–	0.26	–
	K_A^2	9	7	–	–

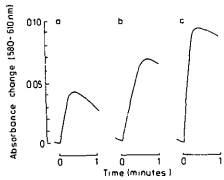

Fig. 7. Absorbance changes caused by addition of 5 mM D-proline (a), 5 mM D-glucose (b) or 5 mM D-proline plus 5 mM D-glucose (c) to luminal membrane vesicles from pars convoluta. The intravesicular medium was 310 mM mannitol and 15 mM Hepes-Tris (pH 7.5), whereas the external medium was 155 NaCl and 15 mM Hepes-Tris (pH 7.5). For further details see legend to Fig. 1.

Competition experiments

The question of whether D-imino acids and their L-isomers (i.e. L-proline and hydroxy-L-proline) are transported by a common transport system or by separate systems was examined as follows. Two substrates, 1 and 2, were added in saturating concentrations either separately or jointly to vesicle-dye suspension and the magnitude of the dye response (ΔA) was compared. Ideally, if the transport processes of substrates 1 and 2 are completely independent of each other, the optical response should be the sum of their individual response according to the following equation:

$$\sum \Delta A = \Delta A_1 + \Delta A_2$$

where $\sum \Delta A$ is the maximal absorbance change observed by simultaneous addition of saturating concentrations of two substrates, and ΔA_1 and ΔA_2 are the maximal absorbance changes induced by individual application of these compounds. However, in reality $\sum \Delta A$ should be less than the sum of ΔA_1 and ΔA_2, since the transport processes of both compounds are driven by the same electrochemical Na^+-tradient and each substrate lowers this gradient resulting in decreased membrane potential across the luminal membrane vesicles. To test this concept a series of competition experiments was carried out with D-proline and D-glucose. Figs. 7a–c show the absorbance changes induced either by individual application of D-proline (Fig. 7a) and D-glucose (Fig. 7b) or simultaneous addition of these compounds (Fig. 7c) to vesicle suspension from pars convoluta. It is seen that both substrates when added alone in the presence of a Na^+-gradient induced maximal absorbance changes by 0.042 and 0.066, but when applied together the maximal absorbance change observed is approx. 0.093 instead of 0.108. Similar experiments were performed using luminal membrane from pars recta (not shown). The results of these experiments showed that D-proline and D-glucose do not share a common transport system.

Alternatively, if the two substrates are transported by the same common transport system, the magnitude of ΔA should be the same as ΔA_1, or ΔA_2 obtained when substrates 1 and 2 are introduced alone in saturating concentrations. In reality, however, the situation may be slightly different because, when dealing with two different compounds 1 and 2, we have to take into account differences in the affinities and maximal transport rates of these substances. A test with D- and L-isomers of proline and hydroxy-proline, which presumably use the same Na^+-dependent (curve 1 in all figures) and Na^+-independent (curve 2 in all figures) transport system(s) present in luminal membrane vesicles from pars convoluta, is shown in Figs. 8a–d. It can be seen that the magnitude of the maximal optical response induced either by simultaneous addition of D-proline and L-proline (Fig. 8b) or hydroxy-D-proline and L-proline (Fig. 8c) or D-proline and hydroxy-D-proline and (Fig. 8d) is approximately the same as caused by the addition of D-proline (Fig. 8a) alone. These results thus suggest that D- and L-isomers of these imino acids probably share common Na^+-dependent and Na^+-independent transport system(s) present in vesicles from pars convoluta. Similar experiments were performed using luminal membrane vesicles from pars recta in the presence of a NaCl gradient (not shown). The results of these experiments also showed that D-and L-proline probably share a common Na^+-dependent transport system located in vesicles from pars recta.

In order to further test the above-mentioned hypothesis the inhibitory effect of D-proline on the uptake of radioactive L-proline by luminal membrane vesicles from pars convoluta as well as from pars recta was examined by the Millipore filtration technique [10]. For the sake of comparison the effect of glycine on the uptake of radioactive L-proline is shown in Fig. 9. Fig. 9A illustrates the effect of two different concentrations

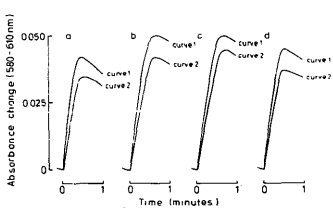

Fig. 8. Absorbance changes caused by addition of 5 mM D-proline (a) or simultaneous addition of D-proline and L-proline (b), or hydroxy-D-proline and L-proline (c) and D-proline plus hydroxy-D-proline (d). Curve 1 and curve 2 in all figures indicate uptake of imino acids in the presence of NaCl and KCl gradients, respectively. For further details see legend to Fig. 1.

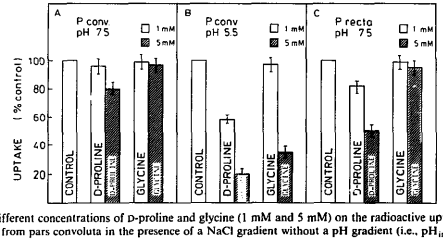

Fig. 9. (A). Effect of two different concentrations of D-proline and glycine (1 mM and 5 mM) on the radioactive uptake of L-proline (100 μM) by luminal membrane vesicles from pars convoluta in the presence of a NaCl gradient without a pH gradient (i.e., $pH_{in} = pH_{out} = 7.5$). (B). Effect of two different concentrations of D-proline and glycine (1 mM and 5 mM) on the radioactive uptake of L-proline (100 μM) by luminal membrane vesicles from pars convoluta in the presence of a pH gradient (i.e., $pH_{in} = 7.5$ and $pH_{out} = 5.5$) without Na^+. (C) Effect of D-proline and glycine (1 mM and 5 mM) on the uptake of radioactive L-proline (100 μM) by luminal membrane vesicles from pars recta in the presence of NaCl gradient without a pH gradient (i.e., $pH_{in} = pH_{out} = 7.5$).

of D-proline and glycine (1 mM and 5 mM) on the Na^+-gradient dependent uptake of radioactive L-proline in vesicles from pars convoluta. In these experiments the intravesicular pH was equal to the extravesicular pH (i.e., $pH_{in} = pH_{out} = 7.5$) and the uptake of 100 μM radioactive L-proline in the presence of a Na^+ gradient and unlabeled D-proline and glycine (1 mM and 5 mM) was recorded. It appears from the figure that the initial uptake (10 s uptake value) of radioactive L-proline into the membrane vesicles was only moderately inhibited by a high concentration (5 mM) of D-proline, whereas the high concentration of glycine has no significant inhibitory effect on L-proline transport. Fig. 9B shows the effect of D-proline and glycine on the H^+-gradient dependent transport of L-proline in the absence of Na^+ ($pH_{out} = 5.5$, $pH_{in} = 7.5$). It is seen that addition of a relatively low concentration of D-proline (1 mM) in the incubation medium decreased the uptake of L-proline to approx. 60% of the control value whereas the low concentration of glycine (1 mM) has no significant effect. Application of a high concentration of D-proline and glycine (5 mM) drastically reduced the H^+-gradient dependent uptake of radioactive L-proline by membrane vesicles from pars convoluta, strongly suggesting that both D-proline and glycine may share the Na^+-independent pathway of L-proline with different affinities in vesicles from pars convoluta. It should be noted here that we have recently demonstrated [12] that the uptake of glycine like that of L-proline can be driven by a H^+ gradient alone (in the absence of Na^+). These findings are in accordance with the present observations. Finally, Fig. 9C shows the effect of D-proline and glycine (1 mM and 5 mM) on the Na^+-gradient dependent uptake of radioactive L-proline (100 μM) in vesicles from pars recta. It is seen that addition of 1 mM and 5 mM D-proline reduces the uptake of L-proline to approx. 80% and 50%, respectively, of the control value whereas the presence of glycine has no effect.

Discussion

Information on the mechanisms of renal transport of D-amino acids acids is lacking in the literature. One of the possible reasons is that the radioactive D-analogs of naturally occurring L-amino acids are not readily available. In the present study the spectrophotometric method [9] has enabled us to study some characteristics of uptake of unlabeled D-proline and hydroxy-D-proline by luminal membrane vesicles from pars convoluta and pars recta of rabbit proximal tubule. In vesicles from pars convoluta, the uptake of D-imino acids was mediated by both Na^+-dependent and Na^+-independent transport processes. The electrical nature of the D-imino acid transport was identified by a positive dye response after addition of these organic compounds and by dependence on membrane potential (negative inside, created by addition of three anions of different known epithelial permeabilities, i.e. $Cl^- > SO_4^{2-} >$ gluconate$^+$). On the basis of these criteria, D-proline and hydroxy-D-proline are transported by electrogenic processes together with various cations. The magnitude of non-electrogenic transport of imino acids cannot be assessed by the spectrophotometric method used in this study. The results shown in Fig. 4 provide indirect evidence for coupling between D-imino acids and H^+ fluxes by the Na^+-independent D-imino acid transporter of membrane vesicles from pars convoluta since addition of D-proline and hydroxy-D-proline in the absence of Na^+ and K^+ depolarized the membrane vesicles and the presence of FCCP (known H^+ ionophore) abolished this transient depolarization of the membrane fragments.

These transport characteristics of D-proline and hy-

droxy-D-proline are similar to those of L-proline uptake properties found in luminal membrane vesicles from pars convoluta of rabbit proximal tubule [1,2], except that only one Na^+-dependent system for D-imino acid transport is detected in the present experiments. For the sake of comparison the K_A and K_m values for L-proline and K_A values for hydroxy-L-proline transport obtained in previous studies [1–3] are given in Table I. It is of interest to note that the K_A and K_m values for L-proline obtained by the spectrophotometric method [1] and Millipore filtration technique [2,3], respectively, are in good agreement, suggesting that these two methods are comparable. Therefore, it is reasonable to assume that the magnitude of transport parameters for D-imino acids obtained in this study by the use of the spectrophotometric method is at least partly valid. However, both in vivo and in vitro kinetic studies are needed to confirm and establish this proposal.

By contrast, the uptake of D-proline in luminal membrane vesicles isolated from pars recta is strictly dependent on Na^+ since no transient depolarization of membrane fragments was ever observed in the absence of Na^+. Another interesting feature of the present study is the observation that addition of hydroxy-D-proline (5 mM) to the vesicle dye suspension did not depolarize membrane vesicles from pars recta, indicating that the D-proline transporter located in this segment of proximal tubule probably has very low affinity for hydroxy-D-proline.

Finally, competition experiments strongly suggest that both the Na^+-dependent and Na^+-independent transport systems of D-imino acids and L-proline are common although with different transport affinities. By contrast, glycine seems to share a Na^+-independent pathway of imino acid transport in vesicles from pars convoluta.

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