Polarized Amino Acid Transport by an Epithelial Cell Line of Renal Origin (LLC-PK₁)

THE APICAL SYSTEMS

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The transport of D-aspartate has been studied in an epithelial cell line from a pig kidney. The amino acid is accumulated by LLC-PK₁ cells without evidence of metabolism. The accumulation against a concentration gradient occurs through a mechanism with several features of a carrier-mediated process. The influx may be accounted for by a saturable Na⁺-dependent and non-saturable Na⁺-independent process. The presence of Na⁺ in the incubation medium increases Vₘₐₓ without affecting Kᵣₑₐₚ. A number of differences in the apparent affinities and specificities allows one to differentiate between this and the acidic amino acid transport system from other tissues. Polarized uptake from either side of the monolayers indicates that the acidic amino acid transport system is preferentially located in the apical membrane of the cultured renal cells. The apical localization of this system clearly contrasts with the basolateral localization of the other three neutral amino acid transport systems reported previously, indicating a high degree of cell polarization. The present study shows a close similarity between the Na⁺-dependent acidic amino acid transport system in LLC-PK₁ cells and the system present in the apical membrane of the proximal tubular cells.

Practically all of the amino acids filtered by the glomerulus are returned to the plasma by absorption along the nephron. Stop-flow clearance (1) and microinjection studies (2) have localized the absorptive site almost exclusively in the proximal tubule. Although the mechanisms involved in the absorption of amino acids across this nephron segment are still imperfectly understood, an extension of Crane's (3) model for the absorption of sugar proposes that the transepithelial transport of amino acids involves at least two sets of transport systems. These systems, which meet the A/ASC/L pattern observed in many other cell types, were a high degree of polarization (12).

The purpose of the present study is to characterize the Na⁺-dependent transport of dicarboxylic amino acid by LLC-PK₁ monolayers. The analysis of the mechanisms involved in this event was performed by studying the uptake of D-aspartate. The selection of this amino acid as a model substrate to explore the acidic amino acid transport system in LLC-PK₁ monolayers was based on two major observations. (a) the D-isomer of the aspartic acid seems to be poorly or not metabolizable at all by the renal tissue (13, 14); (b) selectivity studies indicate that this isomer is also a specific substrate for the acidic amino acid transport system (7, 15-17). The results indicate the presence of a Na⁺-dependent dicarboxylic amino acid transport system in the apical side of the LLC-PK₁ monolayers with similar characteristics to those observed in the proximal tubule.

MATERIALS AND METHODS

Cell Culture and Monolayer Preparation—LLC-PK₁ cells obtained from the American Type Culture Collection (CRL 1392) were maintained by serial passage in 10-cm diameter plastic tissue culture plates (Costar). The cells were fed with Dulbecco's modified Eagle's medium with L-glutamine and 10% fetal bovine serum. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

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1 Terms such as aspartate or Asp refer specifically to the anionic species of this amino acid.
were maintained in an atmosphere of 5% CO₂ in air at 37 °C. When cell growth reached saturation density, subcultures were prepared by using a 0.02% EDTA, 0.05% trypsin solution and plating the cells at a density of 4 × 10⁶ cells/cm². The cells were used between the 209th and 240th passage.

Monolayers on a permeable support were prepared using a polycarbonate filter membrane of 25-mm diameter and 0.8-μm pores (Nuclepore Corp.). The filters were covered with a very thin film of 0.5% collagen dispersion (Ethicon) and applied to a standard microscope slide. The methods for collagen aggregation and stabilization of the collagen-coated membranes were described elsewhere (18). The total thickness of this permeable support is 12 μm.

**Uptake Assays**—The uptake assays were performed in Earle’s balanced saline solution. The composition of this solution is 143 mM Na⁺, 5.36 mM K⁺, 0.8 mM Mg²⁺, 1.85 mM Ca²⁺, 125 mM Cl⁻, 25 mM HCO₃⁻, 1.1 mM PO₄³⁻, 1.1 mM SO₄²⁻. Under an atmosphere of approximately 5% CO₂ in air, the pH of the solution is 7.4. For sodium-free assays, the NaH₂PO₄, NaCl, and NaHCO₃ were replaced by K₂HPO₄, choline chloride, and choline bicarbonate, respectively.

The collagen-coated Nuclepore filter membranes with the monolayers were removed from the microscope slide to provide access to the amino acid from both sides of the membrane. They were washed with PBS and then allowed to take up the labeled amino acid for various times at 25 °C. The uptake medium was Earle's solution, with or without sodium, containing D-[³H]aspartate (1 μCi/ml) and [¹⁴C]inulin (0.25 μCi/ml) as extracellular marker. Unless otherwise specified, d-aspartate uptake was determined under conditions of identical entry rates for the first 5 min of incubation. At the end of each uptake, the filters were washed for 15 s with ice-cold sodium-free Earle’s solution. After dissolving the samples with tissue solubilizer (NCS tissue solubilizer, Amersham Corp.), the radioactivity was measured by liquid scintillation using Dimilume-30 (Packard Instrument Co.) scintillation fluid. Corrections for interstitial trapping were made by measuring the amount of [¹⁴C]inulin associated with each sample. The corrections after the 15-s wash, however, were minimal. Polarized uptake of d-aspartate from the apical or basolateral side was determined on monolayers of LLC-PK₁ cells that had been mounted as a flat sheet between two Lucite chambers. The solutions bathing both surfaces of the tissue were of identical composition with the exception of d-[³H]aspartate, which was added in one experiment to the solution bathing the apical side and in the other to the basolateral side of the cells. After a 5-min uptake period, both solutions were removed, and the membrane was washed in Na⁺-free Earle’s solution at 4 °C for 15 s. The integrity of the monolayer during the 5-min uptake was controlled by measuring the transepithelial electrical resistance as described previously (18). The radioactivity of the sample was determined by liquid scintillation counting techniques as described above.

**Validation of d-Aspartate as a Nonmetabolizable Substrate**—To validate the use of d-aspartate as a nonmetabolizable substrate in the uptake experiments, a group of LLC-PK₁ monolayers were incubated in Earle’s solution containing 0.1 mM d-aspartate and 1 μCi/ml of d-[³H]aspartate. After 1 h of incubation, the monolayers were washed for 15 s with ice-cold sodium-free Earle's solution and placed in 1 ml of ice-cold 5% (w/v) trichloroacetic acid solution for 15 min. The d-aspartate incorporated into protein was determined by measuring the counts/min from tritium retained in the trichloroacetic acid precipitate. The filters with the trichloroacetic acid precipitate were washed three times with ice-cold 5% trichloroacetic acid solution. The radioactivity was measured as described above after dissolving the samples with 0.6 N NaOH. The radioactivity in the trichloroacetic acid-insoluble fraction was determined by liquid extraction of the trichloroacetic acid-insoluble fraction in 0.5 ml of the extraction fluid. The remaining portion of the extraction fluid and a fraction of the incubation medium were used for chromatographic analysis. The extraction fluid and the incubation medium were spotted on a cellulose Eastman chromatogram sheet (20 × 20 cm) and developed for 2 h at 25 °C in a mixture of 1-butanol:acetic acid:water (2:1:1). The amino acid spots were located after exposing the dry plate for 1 h to iodine vapors. The labeled spots were analyzed for radioactivity by separating the individual strips, dividing each strip at 0.5-cm intervals (beginning at the point where each sample was initally spotted), and then placing each piece of the chromatogram sheet into a counting vial for overnight extraction in 1 ml of distilled water. After removing the chromatographic sheet fragments, the radioactivity was determined as described above. With this method, practically all of the radioactivity spotted (≥95%) was recovered as aspartate at the end of the chromatographic analysis.

**Measurement of Cellular Water**—Cell volume measurements were conducted as described before (12). Briefly, the monolayers were equilibrated for 3 h in the presence of 1-[¹⁴C]arabinose (0.25 μCi/ml) and washed for 30 s in ice-cold Na⁺-free Earle’s solution containing 0.1 mM phlorizin. The intracellular water was calculated from the radioactivity retained by the samples and the specific activity of the incubation medium. Cellular water was normalized for the DNA content of each sample.

**DNA Assay**—DNA content was measured as described before (12). Filters were placed in a 5% ice-cold trichloroacetic acid solution for 30 min, washed for 5 min in 0.1 N potassium acetate solution in absolute ethanol, and then air-dried overnight at room temperature. DNA standards were prepared, dissolving calf thymus deoxyribonucleic acid in a 1.0 N ammonium hydroxide solution. The DNA content of the samples and standards was measured in a model 111 Turner fluorometer (G. K. Turner Associates) using a 2 μM 3,5-diaminobenzoic acid dihydrochloride solution.

**Reagents**—All sera, growth media, antibiotics, and trypsin-EDTA solutions were from Gibco (Grand Island, NY). d-[³H]Aspartic acid (specific activity, 19.6 Ci/mmol) and [carboxyl¹⁴C]Inulin (specific activity, 1.91 mCi/g) were purchased from New England Nuclear. [¹⁴C]Arabinose (specific activity, 40 mCi/mmol) was obtained from ICN Chemical & Radiosotope Division. All of the unlabeled amino acids, as well as L-arabinose and calf thymus deoxyribonucleic acid, were obtained from Sigma. 3,5-Diaminobenzoic acid dihydrochloride was obtained from Aldrich.

**RESULTS**

**d-Aspartate Uptake**—The uptake of d-[³H]aspartate by confluent monolayers of LLC-PK₁ cells is shown in Fig. 1. In the presence of Na⁺, the amino acid is accumulated by the monolayer. At a medium concentration of 0.1 mM, the intracellular content reaches a steady state after only a 10-min incubation. Removal of Na⁺ from the incubation medium practically abolishes the uptake of d-aspartate.

An "apparent" intracellular d-aspartate concentration can be calculated from the steady state accumulation values in 0.1 mM. The intracellular water, determined as described under "Materials and Methods," was 119 ± 9 μl/mg of DNA⁻¹ (n = 8). From these values, we calculate an apparent intracellular concentration of 1.1 mM, about 10 times greater than the d-aspartate medium concentration.

**Compartmental Distribution of d-Aspartate**—Studies on the incorporation of d-aspartate into the trichloroacetic acid-insoluble and -soluble fractions during the incubation in Earle’s solution at a concentration of 0.1 mM show that after 1 h of incubation, less than 1% of the radioactivity appears in the acid-insoluble fraction. In addition, chromatographic analysis of the extracts showed a major peak corresponding to d-aspartate, with a lesser peak corresponding to a metabolite of d-aspartate.

**FIG. 1.** Time course of d-Asp uptake. Monolayers of LLC-PK₁ cells were incubated in Earle’s balanced saline solution containing 0.1 mM d-aspartate (○) or without Na⁺ (△) and 10 mM d-aspartate (●). Each point is an average of 12 monolayers ± S.E.
ysis of the extraction fluid reveals a single spot of radioactivity that co-migrates with d-aspartate (results not shown).

**D-Aspartate Washout**—The washout of d-aspartate from the cells is illustrated in Fig. 2. Monolayers equilibrated for 1 h in Earle’s salt solution containing 0.1 mM d-aspartate and 1 μCi/ml of [3H]d-aspartate were transferred to a large volume of Earle’s solution with or without different amino acids. The changes in the d-aspartate content during the washout experiment in the absence of amino acid are fitted by a single first order equation with a rate constant of 0.023 min⁻¹. This constant is apparently not affected by the presence or absence of different amino acids in the incubation medium.

**D-Aspartate Medium Concentration and Initial Uptake Velocity**—Initial uptake velocity was determined during a 5-min uptake over a 200-fold range of d-aspartate concentrations in the incubation medium (0.05 to 10 mM). In the presence of Na⁺, the characteristics of the uptake suggest the presence of both saturable and nonsaturable transport processes (Fig. 3A). In the absence of Na⁺, however, the initial entry rates show only a linear component. The differences between the entry of d-aspartate in the presence or absence of Na⁺ show saturation and are consistent with a single mediated component when analyzed by an Eadie-Hofstee representation (Fig. 3B). A single component can be observed even at concentrations as high as 10 mM (part of the figure not shown). This kinetic analysis indicates that the total entry of d-aspartate into LLC-PK₁ monolayers occurs by at least two components, one that may account for more than 95% of the total uptake and which is Na⁺-dependent with a Kₘ of 0.03 mM and a Vₘₐₓ of 1.98 μmol·h⁻¹·mg of DNA⁻¹ and one which is Na⁺-independent and apparently nonsaturable.

**Medium Sodium Concentration and D-Aspartate Uptake**—The results presented in Fig. 3A indicate that most of the d-aspartate uptake occurs by a Na⁺-dependent process. This effect is quite specific for Na⁺. The rates were 1.40 ± 0.1 in Na⁺, 0.11 ± 0.01 in Li⁺, and 0.01 ± 0.001 mol·h⁻¹·mg of DNA⁻¹ in Cs⁺, K⁺, Rb⁺, or choline. All of these retardations were significant with a p < 0.001. The effect of Na⁺ on the initial entry rate of d-aspartate was measured using Na⁺ concentrations up to 143 mM in the presence of 0.1 mM aspartate (Fig. 4). The apparent Kₐ for the Na⁺ effect was 48 mM. Fig. 5 displays the effect of Na⁺ on the Na⁺-dependent d-aspartate uptake measured using concentrations of d-aspartate from 0.05 to 0.5 mM in the presence of 14 or 143 mM Na⁺. Increases in sodium concentration in the external medium increase Vₘₐₓ from 0.39 to 0.98 μmol·h⁻¹·mg of DNA⁻¹. The apparent Kₐ, however, is not affected as shown by an identical value of 0.03 mM in the presence of 14 and 143 mM Na⁺.

**Effect of Ouabain on the D-Aspartate Uptake and Electrolyte Content**—The effect of ouabain on d-aspartate uptake by LLC-PK₁ monolayers is shown in Table I. The monolayers were first incubated for 60 min in 10⁻⁵ M ouabain. The uptake of d-aspartate at a concentration of 0.1 mM was then determined over a 5-min period in the presence of the same concentration of inhibitor. The results showed that ouabain...
at a concentration that dissipated most of the electrochemical Na⁺ gradient across the cell membrane inhibited 48% of D-aspartate uptake. A further inhibition to 73% was observed on increasing the ouabain concentration to 10⁻³ M. Removal of Na⁺ from the incubation medium seemed to be more effective than the addition of ouabain in blocking the uptake of D-aspartate. This effect, however, was not associated with significant changes in the intracellular electrolyte concentration. The partial reduction of the potassium content in the Na⁺-free medium was associated with a proportional reduction in the cell volume from 119 ± 9 µl-mg DNA⁻¹ to 69.0 ± 6 µl-mg of DNA⁻¹ (n = 6).

Effect of Analogs on D-Aspartate Uptake—Table II shows the effect of several analogs at 10 mM substrate concentration on the D-aspartate uptake by LLC-PKI monolayers. L-Aspartate, D,L-threo-3-hydroxyaspartate, L-cysteine, L-cysteine, and L-glutamate inhibited more than 90% of D-aspartate uptake, to almost the same extent as the absence of Na⁺. D-Methyl-DL-aspartate, N-methyl-DL-aspartate, and D-glutamate produced a partial inhibition, while L-aspartyl-L-phenylalanine, L-asparagine, and succinate apparently did not inhibit the Na⁺-dependent D-aspartate uptake.

Polarized Influx of D-Aspartate in Monolayer of LLC-PKI Cells—Table III shows the influx of D-aspartate at a concentration of 0.1 mM in the medium, with or without Na⁺, when the uptake is measured from the apical or basolateral side of the monolayer. The results indicate that most (88%) but not all of the Na⁺-dependent influx of D-aspartate occurs from the apical side. Still, 12% of the total Na⁺-dependent uptake occurs from the basolateral side. The uptake from the apical side in the absence of Na⁺ is twice as large as the uptake from the basolateral side.

**DISCUSSION**

The present study provides evidence that monolayers of an epithelial cell line of renal origin (LLC-PKI) retain the capacity to accumulate acidic amino acids through a Na⁺-dependent process. A compartmental analysis of D-aspartate accumulation by LLC-PKI cells indicates that less than 1% of the amino acid is incorporated into protein. In addition, chromatographic analysis shows that practically all of the amino acid accumulated by the cell after 1 h of incubation remains as D-aspartate. The uptake in the absence of metabolism together with the possibility to describe the rate of equilibration of the total exchangeable D-aspartate by a single exponential function are evidences that the analog accumulated in the cell is distributed in a single and homogeneous compartment. In addition, the accumulation of amino acid to a steady state value 11 times higher than the amino acid medium concentration indicates the active nature of D-aspartate uptake by LLC-PKI monolayers.
The first order dependence of D-aspartate uptake with respect to Na+ concentration supports the notion that only one Na+ ion may serve as a co-substrate for each molecule of amino acid transported. The increase in Vmax without changes in Ka values suggests that Na+ stimulates D-aspartate uptake in LLC-PK1 monolayers by increasing the velocity of the carrier-solute complex without changing the affinity of the carriers for the ligand (19). These features clearly contrast with those of the Na+-dependent acidic amino acid transport in peripheral nerves (20).

The Na+ gradient hypothesis proposes that the active transport system for certain amino acids in epithelial and nonepithelial cells is driven by the electrochemical Na+ gradient generated by the active extrusion of Na+ through the (Na+-K+)-ATPase (3, 21). The partial inhibition of D-aspartate uptake by ouabain at concentrations that completely inhibit the activity of the membrane-bound (Na+-K+)-ATPase suggests that the uphill transport of this amino acid is energized at least in part by the electrochemical Na+ gradient.

Amino acid transport systems specific for the acidic group have been demonstrated in a number of mammalian tissues, including intestinal mucosa (22), kidney (7), central nervous tissue (23), hepatocytes, and fibroblasts (16). Contrary to what one would expect from the great functional differences between these tissues, the differences in their respective acidic amino acid transport systems are minimal. Inhibition studies using naturally occurring amino acids or chemical analogs have been demonstrated in a number of mammalian tissues, including intestinal mucosa and mammalian tissues, including intestinal mucosa, aspartate uptake by ouabain at concentrations that completely inhibit the activity of the membrane-bound (Na+-K+)-ATPase suggests that the uphill transport of this amino acid is energized at least in part by the electrochemical Na+ gradient.

For instance, like the renal brush-border membrane vesicles (7), but unlike the baby hamster kidney 21-C13 cells (24), the acidic amino acid transport system in LLC-PK1 cells does not exhibit a clear difference in the selectivity between the D- and L-isomers of aspartate. The three systems, however, exhibit a marked preference for the L stereoisomer of glutamate. Similar observations have been reported in cultured rat hepatocytes and human skin fibroblasts where this anomaly has been attributed to the ability of the two carboxylate groups of L-glutamate to bind in the spatial order of α,β for the L-isomer or β,α for the D-isomer and also to bond in the order of γ,α for L-glutamate, but scarcely in the order γ,α for D-glutamate (16). Removal or modification of the α-amino group completely abolishes or reduces the ability of these compounds to be transported via the carrier. Substitution of the α or β hydrogen atoms by a methyl or hydroxyl group, respectively, reduces but does not abolish the affinity of the transport system for the compound. The presence of a second anionic group is also necessary since the amidation but not the substitution of the β carboxyl by a sulfonate group results in a large reduction of the affinity for the transport system. The structural requirements for the substrate in LLC-PK1 monolayers and in renal brush-border membrane vesicles are almost identical, indicating a close similarity between the two.

Polarized uptake from the apical or basolateral side of the monolayer indicates that the amino acid transport system of LLC-PK1 monolayers is preferentially located in the apical aspect of the plasma cell membrane like it is in the renal proximal tubule. This localization, which clearly contrasts with the basolateral localization of three neutral amino acid transport systems reported previously (12), indicates a high degree of cell polarization. Although the apical localization of the Na+-dependent amino acid transport systems has been considered the first step in their transepithelial transport, further studies will be required to determine the functional implications of the apical localization of the acidic amino acid transport system in LLC-PK1 cells.

REFERENCES

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