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LOCALIZATION OF THE Na^+-SUGAR COTRANSPORT SYSTEM IN A KIDNEY EPITHELIAL CELL LINE (LLC PK_1)

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Studies of the localization of the Na^+-dependent sugar transport in monolayers of LLC PK_1 cells show that the uptake of a methyl α-D-glucoside, a nonmetabolizable sugar which shares the glucose-galactose transport system, occurs mainly from the apical side of the monolayer. Kinetics of [^{3}H]phlorizin binding to monolayers of LLC PK_1 cells were also measured. These studies demonstrate the presence of two distinct classes of receptor sites. The class comprising high affinity binding sites had a dissociation constant (K_d) of 1.2 μM and a concentration of high affinity receptors of 0.30 μmol binding sites per g DNA. The other class involving low affinity sites had a K_d of 240 μM with the number of binding sites equal to 12 μmol/g DNA. Phlorizin binding at high affinity binding sites is a Na^+-dependent process. Binding at the low affinity sites on the contrary is Na^+-independent. The mode of action of Na^+ on the high affinity binding sites was to increase the dissociation constant without modifying the number of binding sites. The Na^+ dependence and the matching of K_d for high affinity binding sites with the K_i of phlorizin for the inhibition of methyl α-D-glucoside strongly suggest that the high affinity phlorizin binding site is, or is part of the methyl α-D-glucoside transport system. Binding studies from either side of the monolayer also show that the binding of phlorizin at the Na^+dependent high affinity binding sites occurs mainly from the apical rather than the basolateral side. The specific location of the Na^+-dependent sugar transport system in the apical membrane of LLC PK_1 cells is, therefore, another expression of the functional polarization of epithelial cells that is retained under tissue culture condition. In addition, since this sugar transport almost disappears after the cells are brought into suspension, it can be used as a marker to study the development of the apical membrane in this cell line.

Introduction

Glucose reabsorption across the proximal tubule is an active, saturable, stereospecific and Na^+-dependent process that probably involves at least two transport systems arranged in series, one located at the brush border and the other located at the antiluminal membrane of the epithelial cell [1,2]. The sugar carrier system localized in the renal brush border is specifically susceptible to the competitive inhibitory effect of the phenolic glucoside phlorizin [3,4]. Indeed, two types of binding sites for phlorizin have already been characterized in isolated brush border membrane preparations from rat and rabbit kidney cortex [5,6]. The high affinity phlorizin binding has been characterized as a Na^+-dependent process [5,7]. On the contrary, the low affinity phlorizin binding is Na^+-independent and less specific [5,7]. Since the high affinity phlorizin binding is also inhibited by D-glucose and those analogs that are transported by the glucose transport system [6,8], it has been postulated that the high affinity phlorizin binding occurs to a set of stereospecific membrane sites localized in the brush border of the proximal tubule which are similar

to, if not identical with the glucose transport receptor.

We have recently completed a study [9] which showed that a Na^+-dependent hexose transport system with similar characteristics to that observed in the proximal tubule, was retained in a cultured epithelial cell line from pig kidney (LLC PK_1). In this study, we demonstrated that phlorizin, even at very low concentrations completely inhibited the sugar uptake. Conversely, phloretin at the same low concentration stimulated the sugar accumulation by inhibition of efflux probably at the level of the basal-lateral membrane. Although these observations were consistent with a simple two compartment system where influx and efflux could be measured, the exact location of the sugar entry and exit in the apical and basal-lateral cell border, respectively, needs to be elucidated.

The present paper describes studies on the localization of Na^+-dependent sugar transport in monolayer of LLC PK_1 cells. The uptake of methyl α-D-glucoside from the apical or basal-lateral side was measured and compared as a criteria for localization. Kinetics of phlorizin binding to LLC PK_1 monolayers were also measured. These studies demonstrated the presence of Na^+-dependent 'high affinity' phlorizin binding sites. Binding studies from one or the other side of the monolayer were used as another criteria to localize the Na^+-dependent sugar transport mechanism.

Materials and Methods

Cell cultures and monolayer preparation. LLC-PK_1 cells obtained from the American Type Culture Collection were maintained by serial passages in plastic tissue culture dishes. The cells were fed with Dulbecco's modified Eagle's medium with L-glutamine, supplemented with 10% fetal bovine serum, penicillin (100 Units/ml), streptomycin (100 μg/ml) and Fungizone (0.25 μg/ml). All cultures were maintained in an atmosphere of 5% CO_2/95% air at 37°C. When cell growth reached saturation density, subcultures were prepared using 0.02% EDTA/0.05% trypsin solution.

Monolayers on a permeable support were prepared using a polycarbonate filter membrane with 5 μm pore size and 25 mm diameter (Nucleopore 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 sterilization of the collagen-coated membrane are described elsewhere [10].

Reagents. Methyl α-D-[^{14}C]glucoside, methoxy-[^{3}H]inulin, [^{14}C]inulin and [^{3}H]phlorizin, were purchased from New England Nuclear Corp. Ouabain and phlorizin were obtained from the Sigma Chemical Company. Culture media, fetal bovine serum, trypsin/EDTA solution and antibiotic/antimycotic solution were obtained from Grand Island Biological Co.

Uptake assays. For uptake assays Earle's balanced salt solution was used. Under an atmosphere of approx. 5% CO_2/95% air, the pH of the solution was 7.4. For sodium-free assays, the Na_2HPO_4, NaCl and $NaHCO_3$ were replaced by K_2HPO_4, choline chloride and choline bicarbonate, respectively. The cells grown as monolayers on a collagen-coated Nucleopore filter membrane were removed from the microscope slide to provide access to both sides of the filters. The monolayers were washed with Earle's solution and then allowed to take up the methyl α-D-[^{14}C]glucoside for various times at 37°C. The uptake medium was Earle's solution with or without sodium, containing methyl α-D-[^{14}C]glucoside (0.5 μCi/ml) and methoxy[^{3}H]inulin (1 μCi/ml). At the end of each uptake period, the filters were washed for 15 s with ice-cold Earle's solution. The unidirectional methyl α-D-glucoside influxes were determined under conditions approaching initial entry rates in the first 2–15 min of incubation. The Na-dependent phosphate uptake was measured under similar conditions in the first 5 min incubations. The incubation was performed in Earle's solution with or without sodium and 0.01 mM phosphate concentration containing ^{33}P as monopotassium phosphate (0.5 μCi/ml). After dissolving the samples with tissue solubilizer (NCS tissue solubilizer, Amersham Co.), the radioactivity was measured by liquid scintillation using Dimilume-30 (Packard Inst. Co.) scintillation fluid. Corrections for interstitial trapping were made by measuring the amount of methoxy[^{3}H]inulin associated with each sample. The uptake of methyl α-D-[^{14}C]glucoside by suspended cells was assayed in a volume of 10 ml of cell suspension containing 10^6 cell/ml. Incubations were carried out at 37°C in a 50 ml spinner flask. The uptake was initiated by the addition of 10 ml Earle's balanced salt solution containing the methyl α-D-

[^{14}C]glucoside to yield a final concentration of 10 mM. Aliquots of 500 μl containing $5 \cdot 10^5$ cells were removed at the stated time points and diluted in 15 ml ice-cold Earle's balanced salt solution. The samples were then filtered through Nucleopore filters of 1 μm pore size and 2.5 mm diameter, and washed with 15 ml ice-cold Earle's balanced salt solution. Filtration and washing were finished within 10 s. Cell viability was determined by the trypan blue exclusion test by measuring the plating efficiency or by measuring the intracellular electrolyte concentration. Viability of the suspended cells was higher than 95%. The results were normalized by the DNA content of each sample.

Binding measurements. Binding of [^{3}H]phlorizin was carried out at 37°C. The cells were grown as monolayer on a collagen-coated Nucleopore filter and treated in the same way as in the uptake assay. The binding medium was Earle's solution with or without sodium, containing 0.25–2 μCi/ml [^{3}H]phlorizin and 0.5 μCi/ml [^{14}C]inulin as extracellular marker. At appropriate intervals, the filters were removed from the binding medium and washed for 15 s with ice-cold Earle's solution. The samples were then solubilized and the radioactivity was measured by liquid scintillation as described above. The results were normalized for the DNA content of each sample. Binding of [^{3}H]phlorizin to collagen-coated filter membranes in the absence of cells has a constant value for each concentration used and represents between 2 and 20% of the total content accumulated, depending on the amount of receptors in the tested sample. All experimental values were corrected by this amount.

Evaluation of binding constants. The binding constants of [^{3}H]phlorizin were calculated by assuming that the two-limbed Scatchard plots represent the presence of one specific and one nonspecific binding system [6]. The dissociation constant and the number of binding sites for the nonspecific binding system were calculated from the slope and abscissa intercept, respectively, of the nearly horizontal limb. This limb, obtained by linear least-square fit of the last three data, were subtracted from the composite curve along each diagonal that joins the data with the origin intercept. The different data gave a second straight line that was also fit by lineal least-square analysis. The dissociation constant and the number of binding sites of the specific binding system were calculated from the slope and abscissa intercept, respectively, of this second straight line.

DNA assay. DNA was measured by a modification of the fluorimetric micromethod of Switzer and Summer [11]. Filters were placed in a 5% (w/v) ice-cold trichloroacetic acid solution for 30 min, washed for 5 min in a 0.01 M potassium acetate solution in absolute ethanol and then air-dried overnight at room temperature. DNA standards were prepared, dissolving calf thymus deoxyribonucleic acid (Sigma Chemical Co.) in a 1.0 ammonium hydroxide solution. The DNA of the samples and standards was measured using a 2 M 3,5-diaminobenzoic acid hydrochloride (J.T. Baker Chem. Co.) solution. The fluorimetric determinations were performed in a Model 111 Turner Fluorometer (G.K. Turner Associates) with a 406 nM interference primary filter and a Wratten No. 38 secondary filter. No interference from the collagen-coated Nucleopore filter was observed.

Electrical measurements. The collagen-coated filter with the monolayer was removed from the culture tube and mounted as a flat sheet between two Lucite half chambers with a window area of 3.14 cm^2. To reduce edge damage, the filter was placed between two silicone rubber rings coated with silicone grease as a sealant. Each chamber contained 8 ml solution which was vigorously stirred by a magnetic stirring bar. Transepithelial potential differences were measured with a Keithley model 616 digital electrometer. The electrodes used were pairs of calomel electrodes connected to the bathing solutions by bridges of 3.0 M KCl, immobilized by 4% agar at 1 mm from the tissue. Current conducted by Ag/AgCl electrodes on the opposite sides of the membrane and at the rear of the chamber was measured with a Weston D.C. microammeter, Model 622 (Weston Electrical Instrument Corp., Newark, NJ).

Electrolyte determination. Cells from confluent monolayers or cells removed by trypsin/EDTA treatment were incubated for 5 min in Earle's solution containing L-[^{3}H]glucose (1 μCi/ml) as extracellular marker. After equilibration, the incubation medium was removed and the cells were extracted overnight (18 h or more) in 1 ml 0.1 M nitric acid. Sodium and potassium were measured in the acid extract with an EEL flame photometer using external standards prepared in 0.1 M nitric acid. L-[^{3}H]Glucose was measured in 0.1-ml samples by liquid scintillation count-

ing after neutralization with 0.1 M NaOH. After correction for the extracellular fluid, the results were normalized by the DNA content of each sample.

Cellular water determination. The intracellular fluid volume was estimated from the difference between total water ([^{14}C]urea space) and the extracellular fluid volume (L-[^{3}H]glucose space). After washing with Earle's solution, the cells were gently scraped from the culture dishes with a rubber policeman and centrifuged at 1000 × *g* for 5 min. The cell pellet was suspended in Earle's solution containing [^{14}C]urea (0.5 μCi/ml). After 10 min incubation, L-[^{3}H]glucose was added to the cell suspension to obtain 1 μCi/ml. The incubation was continued for another 5 min to allow the equilibration of L-[^{3}H]-glucose. After 15 min incubation, the cell suspension was centrifuged in a microcentrifuge (Microfuge) for 1 min at 15 000 rev./min. Samples for scintillation counting were taken from the cell pellet and supernatant. The results were expressed by DNA content of each sample.

Results

Polarized uptake of methyl α-D-[^{14}C]glucoside from the apical and basal-lateral side was determined on monolayers of LLC-PK$_1$ cells mounted as a flat sheet between two lucite chambers. Solutions bathing both surfaces of tissue had identical composition. The labeled compound, however, was included in the solution which made contact only with the apical or the basal-lateral side of the cell. After a 15-min uptake period, both solutions were removed and the membrane was washed in ice-cold Earle's balanced salt soluton for 15 s. The integrity of the monolayer during the 15-min uptake was controlled by measuring the transepithelial electrical resistance. A value of

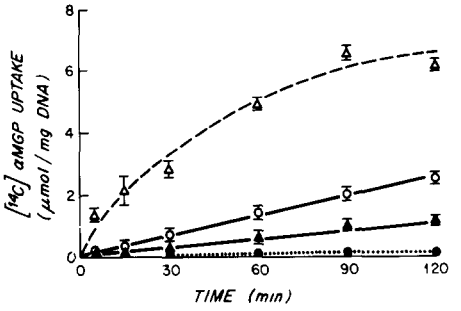

Fig. 1. Effect of phlorizin on the time course of methyl α-D-glucoside (αMGP) by LLC-PK$_1$ monolayer. The monolayers were incubated in 10 ml Earle's balanced salt solution containing 1 mM methyl α-D-glucoside with (●·····●) or without (○———○) 0.01 mM phlorizin, and 10 mM methyl α-D-glucoside with (▲———▲) or without (△- - - - -△) 0.01 mM phlorizin + 0.25 μCi/ml methyl α-D-[^{14}C]glucoside and 1 μCi/ml [^{3}H]inulin as extracellular marker. The monolayers were allowed to take up the methyl α-D-[^{14}C]glucoside for various times at 37°C. Each point is an average of 9–12 monolayers ± S.E.

88 ± 3 Ω · cm^2 (n = 20) was obtained that remained constant during the total experimental period. After dissolving the monolayer in NCS (Amersham Co.), the radioactivity was determined by the liquid scintillation counting technique. Table I indicates that the uptake of methyl α-D-[^{14}C]glucoside determined from the apical side was 0.324 ± 0.002 μmol/h per mg DNA (n = 10), more than 100-times larger than 0.003 ± 0.0003 μmol/h per mg DNA (n = 9) ($P >$ 0.001), the uptake determined from the basal-lateral side of the monolayer.

Effect of phlorizin on the time course of methyl α-D-glucoside uptake

The uptake of methyl α-D-glucoside by LLC-PK$_1$ cells in complete Earle's balanced salt solution in the

TABLE I

POLARIZED UPTAKE OF METHYL α-D-GLUCOSIDE BY LLC-PK$_1$ MONOLAYERS

Uptake from	Concn. of methyl α-D-glucoside (mM)	Uptake ± S.E. (μmol/h per mg DNA)	n	P
Apical side	0.1	0.342 ± 0.002	10	
Basal-lateral side	0.1	0.003 ± 0.00003	9	<0.001

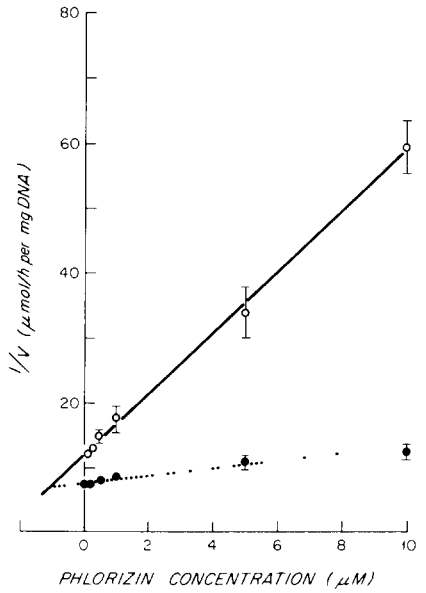

Fig 2. Dixon plot of methyl α-D-glucoside uptake at concentrations of 0.1 mM (o———o) and 1 0 mM (• ·· ·•) in the presence of different concentrations of phlorizin. V is the methyl α-D-glucoside uptake expressed as μmol/h per mg DNA The phlorizin concentration is expressed in μM. The line was fitted to the data using least-square determinations. From these data, the apparent K_1 value calculated for phlorizin was 1.1 μM. Each point is an average of 6–10 determinations ± S.E

absence or presence of phlorizin is illustrated in Fig. 1. In the absence of phlorizin, there is a continual accumulation of the sugar. At a concentration of 1 mM, the rate of uptake remains constant up to 120 min. Increasing the sugar concentration in 10 mM increases the uptake velocity, which however, declines with the incubation time. In the presence of phlorizin, the uptake of sugar is greatly reduced.

The 'apparent' intracellular concentration of the tested sugar was calculated after correction for the extracellular space, using intracellular water values of 114 ± 3.0 μl/mg DNA (n = 6) and 124 ± 4.3 μl/mg DNA (n = 6) measured in the presence of 1 and 10 mM α-methyl-D-glucoside, respectively. The values after 1 h accumulation without phlorizin were 12.1 and 39.5 mM in 1 and 10 mM sugar medium concentrations, respectively. Phlorizin at a concentration of 0.01 mM reduced these values to 0.60 and 4.58 mM, respectively.

Phlorizin concentration and inhibition of methyl α-D-glucoside uptake

The inhibition of methyl α-D-glucoside uptake was determined over a 100-fold range of phlorizin concentration in the incubation medium. The uptake of methyl α-D-glucoside and phlorizin concentration were plotted in Fig. 2 by the method of Dixon. The straight lines were fitted to the data using least-square determinations. From these data, the apparent K_1 value calculated for phlorizin was 1 1 μM.

Inhibition of methyl α-D-glucoside uptake compared to [^{3}H]phlorizin binding measured under equilibrium conditions

Fig. 3 shows the relationship between percentage inhibition of methyl α-D-glucoside uptake and bound phlorizin after 60 min incubation. Different amounts of bound phlorizin were obtained using different concentrations of [^{3}H]phlorizin in the range 0.14–50 μM.

The percentage inhibition of methyl α-D-glucoside uptake was determined simultaneously by comparing the uptake of methyl α-D-[^{14}C]glucoside in the presence or absence of [^{3}H]phlorizin during 60 min. An increase in the bound [^{3}H]phlorizin was associated with an increase in the inhibition of methyl α-D-glucoside uptake. The uptake inhibition was almost complete when the bound phlorizin reached 3 μmol/g

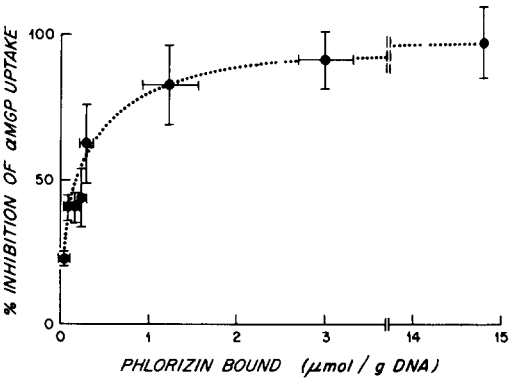

Fig 3 Inhibition of methyl α-D-[^{14}C]glucoside uptake compared to [^{3}H]phlorizin binding measured under equilibrium conditions. Binding of phlorizin and uptake of methyl α-D-glucoside (αMGP) at a concentration of 0.1 mM were carried out after 60 min incubation as described in the text. Each point is an average of 12 determinations ± S E

DNA which corresponded to a phlorizin concentration of 10 μM. However, an increase in phlorizin concentration to 50 μM induced a further increase in the bound phlorizin.

Time course of total [^{3}H]phlorizin binding

Fig. 4 shows the rate of total [^{3}H]phlorizin binding to monolayers of LLC-PK$_1$ cells at 37°C. Binding at a concentration close to the K_i (0.85 μM) is essentially complete within 5 min. However, after that, an additional increase is observed, but at a much lower rate. The binding between 5 and 30 min increases only 25% compared with the binding in the first 5 min.

Rapid high affinity [^{3}H]phlorizin binding

The data presented in Fig. 4 suggest the presence of two different phlorizin binding sites with different rate constants. An increase in the number of low specificity binding sites associated with an increase in the incubation time has been reported [12]. Experiments were then designed to analyze the relationship between the two types of binding sites characterized by different rate constants with those referred to in the literature as high and low affinity binding sites. The results are illustrated in Fig. 5, plotted in the form of the Scatchard equation [13] so that the slopes represent the affinities and the abscissa intercepts represent the number of sites. In the range of phlorizin concentration, 0.143–50 μM, two sets of binding sites were identified. The apparent dissociation constant calculated from the slopes for the high and low affinity binding sites were 1.2 and 240 μM, respectively, both within the reported range [5–8]. The constant for the high affinity binding sites was not apparently modified by the incubation time between 5 and 60 min. The number of high affinity binding sites also remained approximately constant with values of 0.30 μmol/g DNA and 0.35 μmol/g DNA for 5 and 60 min, respectively. The number of low affinity binding sites was reduced from 177 μmol/g DNA to 12 mol/g DNA when the incubation time was diminished from 60 to 5 min. An incubation time of 5 min was then chosen for most experiments in order to reduce the number of nonspecific binding sites.

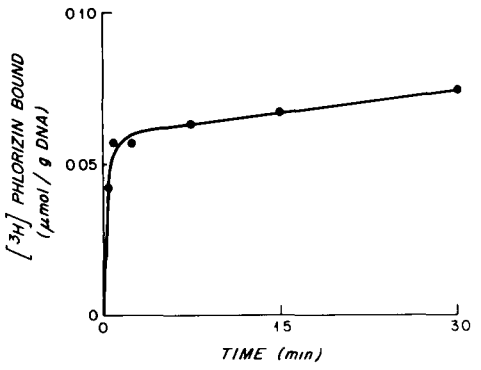

Fig. 4 Time course of [^{3}H]phlorizin binding to monolayers of LLC-PK$_1$ cells. Binding was carried out as described in the text. The phlorizin concentration was 0.85 μM Each point is the mean of three determinations.

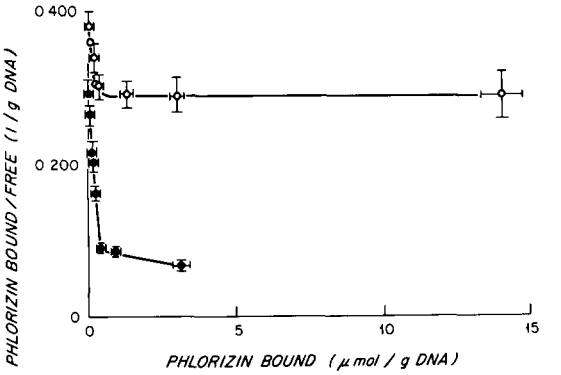

Fig. 5. Scatchard plot of [^{3}H]phlorizin binding to monolayer of LLC-PK$_1$ cells after 5 (●———●) or 60 (○———○) min incubation. Each point is the mean of 8–16 determinations ± S.E.

Effect of Na^+, ouabain and medium osmolarity on total phlorizin binding

The data in Table II show that the binding of [^{3}H]phlorizin at a concentration of 0.56 μM was inhibited about 40% by the removal of Na^+ from the incubation medium. An inhibition of about 20% was also observed in the presence of 5 μM ouabain. The binding in the absence of Na^+ or presence of 5 μM ouabain in the incubation medium was performed during a 60-min period. The binding of [^{3}H]phlorizin at a concentration of 0.28 μM was not affected by the medium osmolarity after 5 min incubation.

Effect of Na^+ on high affinity phlorizin binding sites

A Scatchard plot of equilibrium phlorizin binding

TABLE II

EFFECTS OF DIFFERENT INCUBATION MEDIA ON PHLORIZIN BINDING

Condition	Phlorizin concn. ($\times 10^{-6}$ M)	Phlorizin binding ± S E (μmol/g DNA)	n	P
Control medium	0.56	0.073 ± 0 005	9	
Na^+-free medium	0.56	0.044 ± 0.003	9	<0.001
5 μM ouabain	0.56	0.059 ± 0.003	9	<0.05
Control medium (300 mOsm)	0.28	0.032 ± 0.002	7	
Hyperosmolar medium (600 mOsm)	0.28	0.029 ± 0.001	7	<0.6

in the presence or absence of 142 mM Na^+ is illustrated in Fig. 6. In the presence of Na^+ and in the range of phlorizin concentration 0.143–50 μM, two types of binding sites were identified. The Scatchard plot in the presence of Na^+ was replotted from Fig. 5. The K_d value for [^{3}H]phlorizin binding to low affinity binding sites was essentially the same in the presence (K_d 240 μM) or absence of Na^+ (K_d 200 μM). On the contrary, the K_d value for [^{3}H]phlorizin binding to high affinity binding sites was clearly increased from 1 to 10 μM in the absence of Na^+ in the incubation medium. The mode of action of Na^+ on the binding of phlorizin at high affinity binding sites was to increase the apparent K_d, without modifying the number of binding sites, as shown by the same abscissa intercept in Fig. 7.

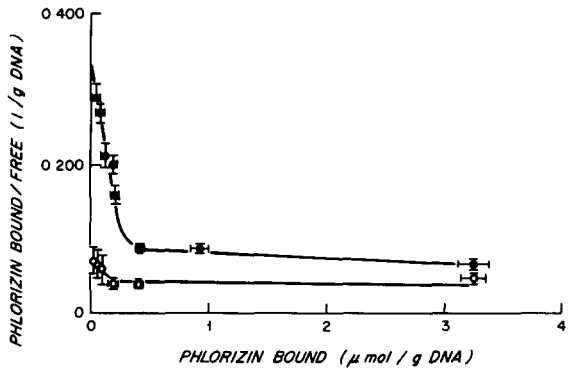

Fig. 6 Scatchard plot of [^{3}H]phlorizin binding to monolayer of LLC-PK$_1$ cells after 5 min incubation in the presence (●———●) or absence (○———○) of 142 mM Na^+. Each point is the average of eight determinations ± S.E.

TABLE III

EFFECT OF PHLORIZIN ON THE Na-DEPENDENT PHOSPHATE UPTAKE BY LLC-PK$_1$ MONOLAYERS

Condition	Phosphate uptake ± S.E. (μmol/h per mg DNA)	n	P
Control	97.3 ± 10 5	7	
Phlorizin (10 μM)	92.1 ± 7.0	7	<0.7

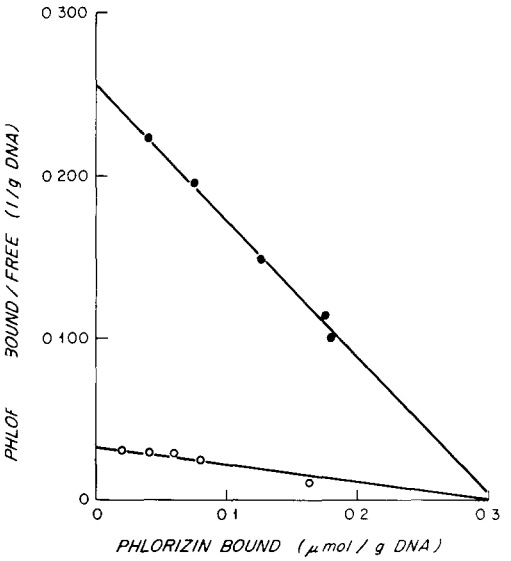

Fig. 7 Scatchard plot of [^{3}H]phlorizin binding to high affinity binding sites in the presence (●———●) or absence (○———○) of 142 mM Na^+. The high affinity binding sites were obtained as the difference between the composite curve and the amount of phlorizin bound at low affinity binding sites from Fig. 6. Additional values in the absence of Na in the high affinity range not shown in Fig. 6 are also included.

Localization of the high affinity phlorizin binding sites

The localization of the Na^+-dependent high affinity phlorizin binding sites on the apical and/or basal-lateral side of LLC-PK_1 monolayers was studied by mounting the monolayers as a flat sheet between two Lucite half chambers. Solutions bathing both sides of the monolayers had identical composition. [^{3}H]-Phlorizin at a concentration of 0.84 μM was added to the solution which made contact only with the apical or the basal-lateral side of the cell. A similar concentration of cold phlorizin was applied to the contralateral side. The integrity of the monolayer during the binding experiments was controlled by measuring the transepithelial electrical resistance. A value of 82 ± 3 $\Omega \cdot cm^2$ (n = 12) was obtained that remained constant during the experimental period. After 5 min of binding, both solutions were removed and the monolayer treated as described in Materials and Methods. Table IV shows the [^{3}H]phlorizin binding in the presence or absence of Na^+ performed from the apical or basal-lateral side of the monolayer. In the presence of Na^+, the binding from the apical and basal-lateral sides was almost the same. In the absence of Na^+, however, a 60% reduction was observed when the binding was performed from the apical but not from the basal-lateral side of the monolayer ($P < 0.001$).

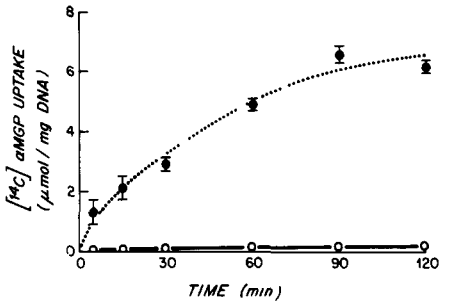

Fig. 8. Uptake of methyl α-D-glucoside (α MGP) by monolayer (• · · · •) or suspended (o———o) LLC-PK_1 cells. The incubations were carried out in Earle's balanced salt solution containing 10 mM methyl α-D-glucoside + 0.25 μCi/ml methyl α-D-[^{14}C]glucoside and 1 μCi/ml [^{3}H]inulin as extracellular marker. Each point is an average of 6–12 samples ± S.E.

Time course of methyl α-D-glucoside uptake by monolayers or suspension of LLC-PK_1 cells

Fig. 8 shows the time course of methyl α-D-[^{14}C]-glucoside uptake by LLC-PK_1 cells grown as con-

TABLE IV

Na-DEPENDENT [^{3}H]PHLORIZIN BINDING FROM THE APICAL OR BASAL-LATERAL SIDE OF THE MONOLAYERS

Binding from [a]	Na in the incubation media	Binding ± S.E. (μmol/g DNA)	n	P
Apical side	Present	0.0198 ± 0.0011	12	
	Absent	0.0074 ± 0.0006	12	<0.001
Basal-lateral side	Present	0.0218 ± 0.0012	10	
	Absent	0.0190 ± 0.0015	10	<0.2

[a] Phlorizin concentration = 0 85 μM.

TABLE V

ELECTROLYTE COMPOSITION OF CONFLUENT MONOLAYER AND SUSPENDED LLC-PK_1 CELLS

Suspended cells were prepared by trypsin/EDTA treatment of confluent monolayers during 15 min.

Time after plating (h)	Na^+ content ± S.E. (μmol/mg DNA)	K^+ content ± S.E. (μmol/mg DNA)	n
0	1.86 ± 0.18	21.0 ± 0.5	12
3	1.97 ± 0.12	19.3 ± 0.9	12
6	1.31 ± 0.09	19.1 ± 0.9	12
24	2.26 ± 0.10	21.7 ± 0.7	12

fluent monolayers or maintained in suspension. We observe that after the cells are brought into suspension, the uptake of sugar is greatly reduced. In addition, Table V shows that suspended cells have an electrolyte composition similar to the confluent monolayer 24 h after plating. This result indicated that the decrease in the sodium-dependent sugar uptake by the suspended cells is not associated with changes in the electrolyte distribution across the cell membrane.

Discussion

Previous results indicated that methyl α-D-glucoside, a nonmetabolizable sugar, which shares the glucose-galactose system in kidney cells is actively transported by monolayers of LLC-PK_1 cells derived from pig kidney [9,14]. This sugar transport is mediated through a Na^+-dependent system which conforms to the kinetics of a substrate-saturable process with similar characteristics as observed in the apical membrane of the proximal tubular cells [14, 15]. The Na^+-sugar cotransport system is one of the most representative of the large number of functional markers associated with the apical membrane of epithelial cells. In order to take advantage of this system to study the molecular events associated with the development of the apical barrier, we must demonstrate that this transport system is also localized in the apical membrane of the LLC-PK_1 cells.

The polarized uptake of methyl α-D-glucoside shown in Table I could be a good indication of the apical localization of the Na-dependent transport system, providing that the unstirred layers do not play an important role in this polarized uptake. Since the LLC-PK_1 monolayers are formed using a collagen-coated Nucleopore filter as a support, it can be argued that the different uptake from the apical and basal-lateral side is due to a thick unstirred layer produced by the presence of this support on the basal-lateral side. However, the thickness of the collagen-coated Nucleopore filter is 12 μm [10], about half of the thickness of the unstirred layer calculated from the time course of the electrical potential difference [16] and short circuit current [17] obtained in thoroughly stirred preparations. A restriction in the diffusion of the sugar through this support is also ruled out by results from a previous study which showed that the collagen-coated Nucleopore filters behave the same as a layer of fluid of similar thickness [10].

If we assume a three-compartment system for the active transepithelial sugar transport, as was proposed by Crane [18] for intestine, the large difference between the uptake from the apical and basal-lateral sides indicates that the rate-limiting barrier in the transcellular route is localized in the basal-lateral membrane.

The electrical resistance across the monolayer measured 7 days after plating was 88 $\Omega \cdot cm^2$. Higher values have been reported for monolayers obtained with the same cell line [15,20]. The differences, however, probably represent differences in the experimental conditions used in these studies. For instance, values similar to those reported [15,19] have been observed at different times after plating [20]. Since these changes have been associated with changes in cell density of the monolayer [20], it is possible that the differences observed represent differences in cell density, rather than differences in the intrinsic properties of the tight junctions.

The uptake of methyl α-D-glucoside by LLC-PK_1 cells is completely blocked by the presence of phlorizin at a concentration of 10 μM (Fig. 1), in agreement with the inhibitory effect of the glycoside on the active translocation of sugar found in kidney [21] and intestine [22]. Since phlorizin is an inhibitor of the oxidative phosphorylation in renal tissue [23], the inhibition of the sugar uptake induced by the glycoside could be mediated through the dissipation of the sodium electrochemical gradient. However, this metabolic effect requires does 10-times higher than the maximal dose used in the present study. Furthermore, Table III shows that phlorizin at a concentration that completely inhibited the sodium-dependent sugar uptake does not modify the sodium-dependent phosphate uptake observed in this cell line [24]. From the kinetic data shown in Fig. 2, the apparent K_i calculated for phlorizin was 1.1 μM within the reported range [25,26]. Since phlorizin completely inhibits this sugar transport without crossing the cell membrane [27,28], it is postulated that phlorizin interacts with and binds to the proposed sugar carrier. In agreement with this idea, no changes in the phlorizin binding were observed with the increase in the medium osmolarity induced by an impermeable solute (Table II). This indicated that the

glycoside accumulated by the monolayers during 5 min incubation represents bound phlorizin and not uptake into the cellular compartment. However, especially after a 60 min incubation (see later), it is possible that the so-called low affinity binding observed at higher phlorizin concentrations represents net uptake into the cell [5]. Fig. 3 shows that there is a direct relationship between the bound phlorizin and the inhibition of methyl α-D-glucoside uptake by LLC-PK_1 monolayers. This figure also shows that the sugar uptake is completely inhibited when the bound phlorizin is 3 μmol/g DNA. This amount corresponds to a concentration of 10 μM phlorizin in the medium. An increase in the phlorizin concentration of 50 μM produces a further increase in the bound phlorizin without, obviously, a further increase in the inhibition of the methyl α-D-glucoside uptake. This result suggests the presence of binding sites not associated with the inhibition of the sugar uptake. The results shown in Fig. 5 indicate the presence of two binding systems for phlorizin, comprising high or low affinity sites. The high affinity binding sites have a K_d value of 1.2 μM which is not modified by the incubation time between 5 and 60 min. The number of these high affinity binding sites also remain constant with a value of 0.30–0.35 μmol/g DNA. The low affinity binding sites, however, have a K_d value of 240 μM. The number of these binding sites increase from 12 to 177 μmol/g DNA if the incubation time varies between 5 and 60 min, respectively. This indicates that after a longer period of incubation, the high affinity binding sites were submerged by a massive amount of low affinity binding sites. Similar phenomena have been observed in isolated brush border membranes from rabbit intestine [12]. The presence of two binding sites in LLC-PK_1 monolayers is in agreement with the recent observations in membranes prepared from brush border of renal epithelial cells, which indicate the presence of two binding systems for phlorizin with similar affinity constants for high and low affinity binding sites as reported above.

Phlorizin binding to brush border membranes was found to be a sodium-dependent process [5–7] also influenced by the electrochemical Na^+ gradient [29]. Results presented in Table II agree with these observations. When the Na^+ in the incubation media was replaced by choline, a 40% inhibition in the phlorizin binding was observed. A concentration of 5 μM ouabain also inhibits the phlorizin binding. This inhibition is probably due to the dissipation of the electrochemical gradient for Na^+ produced by inhibition of the ($Na^+ + K^+$)-ATPase localized in the basolateral membrane [30]. In renal brush border membranes, however, in which no Na^+ electrochemical gradient was present, inhibition of the phlorizin binding by ouabain was not observed [5].

The effect of Na^+ on phlorizin binding (Figs. 6 and 7) shows that the binding of phlorizin at high affinity binding sites was a Na^+-dependent process. In contrast, Na^+ was not required for binding at low affinity binding sites. These results are in total agreement with those obtained in brush border membranes [5–7] that show a Na^+ dependence only for the high affinity binding sites. When the amount of phlorizin bound at low affinity binding sites is substracted from the composite curve, the difference represents the so-called 'high affinity binding sites'. Fig. 7 shows the Scatchard plot for the high affinity binding sites obtained in the presence or absence of Na^+ in the incubation media. These results indicated that the mode of action of Na^+ on the binding of phlorizin at high affinity sites was to increase the affinity of phlorizin to the receptor. This is shown in Fig. 7 by the increase in the slope of the high affinity binding sites produced by the presence of Na^+. The K_d values calculated from the slopes were 1.2 and 10 μM in presence or absence of Na^+, respectively. The number of binding sites, however, was not affected by the presence or absence of Na^+, as shown by the same abscissa intercept in the Scatchard plot of Fig. 7. Results obtained in isolated luminal membranes of renal proximal tubule show that Na^+ decreases the apparent K_i for phlorizin, while the apparent V of binding was not altered [5]. These results are in total agreement with the effect of Na^+ reported here.

The active transport of methyl α-D-glucoside by LLC-PK_1 monolayers has been well characterized as a Na^+-dependent, phlorizin-sensitive process [9]. The present study also shows that [^{3}H]phlorizin binds at high affinity binding sites in LLC-PK_1 cells with a K_d value of 1.2 μM which matches the K_i value of 1.1 μM for the inhibition of methyl α-D-glucoside transport. Since the K_m for the sugar [9] and the K_d for phlorizin also change simultaneously when altering the experimental conditions, we can suggest that the Na^+-dependent high affinity phlorizin binding sites

are related to the methyl α-D-glucoside transport unit. Results shown in Table IV indicate that the Na^+-dependent phlorizin binding sites are mainly localized in the apical side of the LLC-PK_1 monolayer. These results together with the polarized uptake of methyl α-D-glucoside indicate that the Na^+-sugar cotransport mechanism is mainly localized in the apical membrane of LLC-PK_1 cells, in total agreement with similar studies performed in brush border membranes of kidney proximal tubular cells [5–7,29], and with the relationship observed between development of the microvillous surface and uptake of methyl α-D-glucoside by LLC-PK_1 monolayers [14]. In addition, since this sugar transport almost disappears after the cells are brought into suspension (Fig. 8) probably by a protease-induced damage of the membrane-bound system [6], it can be used as an excellent functional marker to study the events in the development of the apical membrane, during the reconstruction of the monolayer from isolated LLC-PK_1 cells.

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