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SODIUM-DEPENDENT TRANSPORT OF PHOSPHATE IN LLC-PK$_1$ CELLS

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Transport of phosphate has been studied in subconfluent monolayers of LLC-PK$_1$ cells. It was found that this transport system shows similar characteristics to those observed in the kidney. Uptake of phosphate is mediated by a Na$^+$-dependent, substrate-saturable process with an apparent K_m value for phosphate of 96 ± 15 μmol/l. Kinetic analysis of the effect of Na$^+$ indicated that at (pH 7.4) two sodium ions are cotransported with one HPO_4^{2-} ion (Hill coefficient 1.5) with an apparent K_m value for sodium of 56 mmol/l. P_i uptake is inhibited by metabolic inhibitors (ouabain and FCCP). In the pH range of 6.6 of 7.4 P_i uptake rate does not change significantly, indicating that both the monovalent and the divalent form of phosphate are accepted by the transport system. It is suggested that phosphate is transported by LLC-PK$_1$ cells together with sodium (2 Na$^+$: 1 HPO_4^{2-}) in an electroneutral manner down a favourable sodium gradient.

Introduction

Over the last decade, it has become apparent that epithelial cells derived from a number of species and organs form polarised epithelia under standard cell culture conditions. Furthermore a large body of data has now been presented describing the retention of membrane transport systems by these epithelia which are characteristic of the parent tissue [1]. One such epithelium, LLC-PK$_1$ originally derived from pig kidney [2] displays many of the features of renal proximal tubule epithelium.

In addition to displaying several 'marker enzymes' for the proximal tubules [3], several laboratories have reported that LLC-PK$_1$ cells possess a sodium-dependent glucose transporter with characteristics similar to that found in renal proximal tubules [3–5]. In a series of further experiments the glucose transporter was shown to be localised to the apical membrane of LLC-PK$_1$ [6], and displays similar kinetics and substrate specificity as the transport system described in renal brush-border membrane vesicles [6,7]. Recently a sodium-dependent amino acid transport system, with properties similar to that found in the renal proximal tubule, has also been described [8,9].

The present study describes the transport of inorganic phosphate by subconfluent monolayers of LLC-PK$_1$. The major result is that phosphate is transported by a sodium-dependent transport mechanism that displays many of the features of the phosphate transport system found in renal proximal tubules [10,11]. This result not only reinforces the usefulness of LLC-PK$_1$ cells as a model epithelium for the proximal tubule, but also provides an ideal model with which to study regulation of phosphate transport.

Materials and Methods

(i) Cell culture

LLC-PK$_1$ pig kidney cells [2] were obtained from Flow Laboratories, Irvine, Scotland at 145 serial passages. Cells were maintained in serial culture in 120 cm^2 (growth area) Roux flasks in

Dulbecco's modification of Eagle's minimum essential media (DMEM) supplemented with 5% (v/v) foetal calf serum, 5% (v/v) donor horse serum, 1% (v/v) non essential amino acids and penecillin (100 I.U./ml)/streptomycin (100 μg/ml) as an antibiotic. Cells were routinely subcultured by trypsinisation (0.25% trypsin in a Ca^{2+}/Mg^{2+} free Earle's salt solution containing 2 mmol/l EDTA). The split ratio was 1:10. For experimental purposes LLC-PK_1 cells were grown as subconfluent cell monolayers in plastic petri dishes (Nunc, 5 cm diameter). These were prepared by seeding at $1 \cdot 10^5$ cells per plate in 3 cm^3 of complete media. Uptake measurements were routinely made after 3 days of growth while the cell monolayers were still subconfluent (final cell density was $1 \cdot 10^6$–$2 \cdot 10^6$ cells per plate).

(ii) Phosphate uptake assays

All experiments were performed in the following media (in mmol/l): 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$, 14 Tris base plus the substrate (KH_2PO_4 or mannitol) at the desired concentration. The pH was adjusted with concentrated HCl. A sodium-free media was made by replacing NaCl with choline chloride.

To study phosphate uptake into LLC-PK_1 cells the monolayers were first washed briefly with uptake media (substrate-free) and flux initiated by the addition of 2 cm^3 of media containing labelled substrate (either $KH_2{}^{32}PO_4$ or [^{3}H]mannitol) at a specific activity of 1 μCi/cm^3. Uptake was usually measured at 25°C over a 5 min incubation period (see Fig. 1). The plates were continuously shaken throughout the uptake period (100 cycles/min). $^{32}PO_4$ uptake was terminated by removing the uptake media and washing the cells with an excess of cold (4°C) substrate-free media. Isotope was then extracted by solubilising the cells in 1 cm^3 2% SDS, subsequently an aliquot was counted by liquid scintillation counting.

(iii) Presentation of data

Phosphate uptake is expressed as pmol phosphate per mg protein and usually per 5 min. The protein content of a batch of identical plates, treated in the same way as those for $^{32}PO_4$ uptake, was determined by the method of Lowry et al. [12] using bovine serum albumin as the standard. All uptake data were corrected with a blank obtained from an incubation period of less than 10 s.

The data are expressed as means ± S.D. and analysed using a Student's *t*-test (unpaired means solution). Regression lines were calculated by a least-squares method.

(iv) Reagents

Cell cultures and all cell culture media were obtained from Flow Laboratories, Irvine, Scotland. Radiochemicals were purchased from New England Nuclear Corp., Boston. FCCP was from Fluka, Switzerland. Analytical grade choline-chloride was obtained from Merck (F.R.G.) and all other reagents were of the highest commercially available purity.

Results

The uptake of phosphate by subconfluent cell monolayers of LLC-PK_1 cells either in the presence or absence of sodium (choline replacement) is shown in Fig. 1. In the absence of sodium phosphate uptake was very much less than in the presence of sodium, and mirrored that of [^{3}H]mannitol (mannitol uptake was the same plus and minus sodium). Since it can be assumed that mannitol is not transported via a specific carrier mechanism, phosphate uptake in the absence of

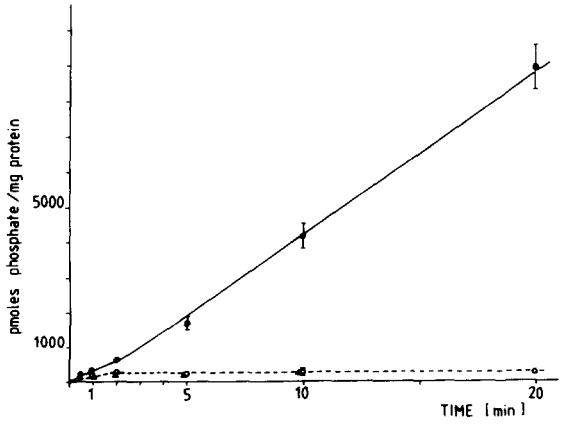

Fig. 1. Time dependence of phosphate uptake by LLC-PK_1 cells. Monolayers were incubated with Na^+ (●) or without Na^+ (○) (NaCl replaced by choline chloride) plus 0.1 mmol/l $K_2H^{32}PO_4$ (1 μCi/ml). Uptake of [^{3}H]mannitol (▲) (0.1 mmol/l) was determined either in the presence or in the absence of sodium. Each point represents the mean ± S.D. of five determinations.

extracellular sodium might represent a passive, diffusive uptake of phosphate into the cells, or less likely trapping of isotope in an extracellular space. After an initial lag phase of about 1–2 min, Na^+-dependent phosphate uptake is linear over the next 20 min ($r = 0.99$). Although the initial lag phase is a consistent feature of phosphate uptake into LLC-PK_1 cells, we have no explanation of its reason.

Table I illustrates the cation dependence of phosphate uptake by LLC-PK_1 cells. It is clear from this data that phosphate uptake was specifically dependent upon sodium. Compared to choline, phosphate uptake was stimulated by a factor of about 10 by sodium. Similar to many other sodium-dependent transport mechanisms (see for example, Refs. 13 and 14) Li^+ was the only other cation which could partially substitute for sodium. Phosphate uptake in the presence of either Cs^+ or K^+ did not differ significantly from that in the presence of choline. Although potassium ions themselves may not substitute for Na^+, a reduced phosphate uptake in a K^+ media may also result from unspecific alterations in driving forces, e.g. changes in membrane potential.

Fig. 2A shows that phosphate uptake into the cell at different phosphate concentrations consists of two components: a saturable sodium-dependent component and a linear sodium-independent component. Analysis of the sodium-dependent component of uptake by a Lineweaver-Burk plot (Fig. 2B) gave an apparent K_m value for phosphate of 96 ± 15 μmol/l and an apparent V_{max} of 4165 ± 700 pmol phosphate/mg per 5 min. In contrast to other systems [15] no evidence for a second saturable component was found when substrate concentration was increased by up to 5 mmol/l (data not shown).

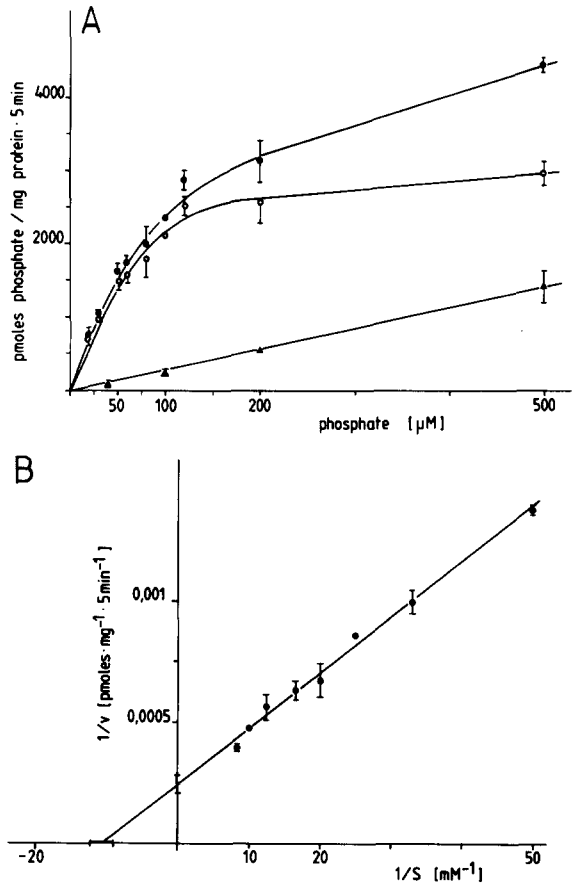

Fig. 2. Kinetics of P_i uptake by LLC-PK_1. (A) Net-sodium dependent P_i uptake (○) was calculated as the difference between P_i uptake in the presence of 137 mmol/l NaCl (●) and P_i uptake in the presence of 137 mmol/l choline chloride (▲). (B) Lineweaver-Burk plot of Na^+-dependent P_i uptake. The apparent K_m and V_{max} calculated were 96 ± 15 μmol/l and 4165 ± 700 pmol/mg per 5 min. Each point represents the mean ± S.D. of four determinations.

TABLE I

EFFECT OF DIFFERENT CATIONS ON PHOSPHATE UPTAKE BY LLC-PK_1 CELLS

Salt [a]	Total P_i uptake [b] (pmol/mg/5 min)	% of uptake in choline medium
Choline-Cl	336 ± 69	
NaCl	3931 ± 175	1170 ($p < 0.0001$)
LiCl	1402 ± 106	417 ($p < 0.001$)
CsCl	744 ± 285	220 ($p < 0.2$)
KCl	680 ± 64	202 ($p < 0.01$)

[a] Each salt was used at a concentration of 137 mmol/l.
[b] Each value represents the mean ± S.D. of four determinations.

The effect of various concentrations of NaCl upon the initial uptake velocity of 0.1 mmol/l phosphate is shown in Fig. 3A. Over the concentration range tested (0–137 mmol/l) increased phosphate uptake was observed with increased concentrations of sodium, at physiological sodium concentrations phosphate uptake was not yet saturated. At low sodium concentrations the curve tended to be non-hyperbolic. Analysis of these data by Hill analysis (not shown) resulted in a Hill

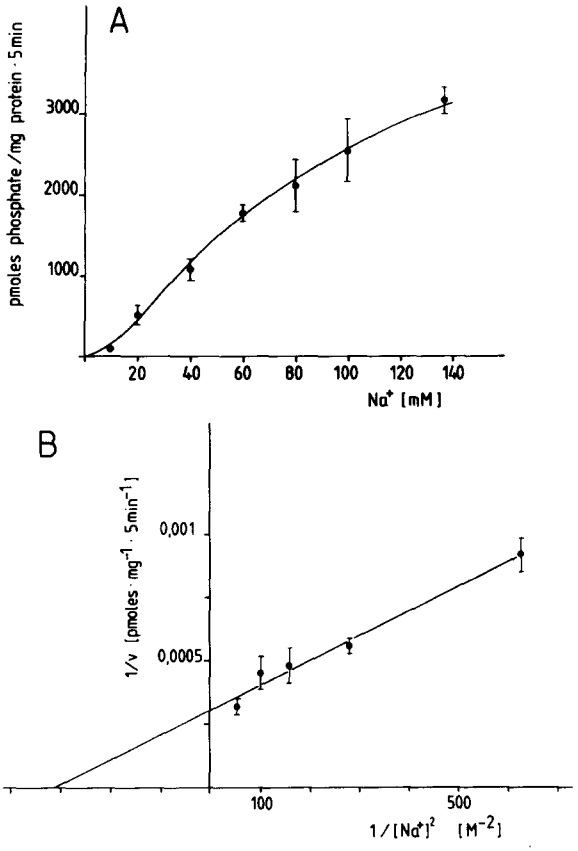

Fig. 3. Influence of sodium on P_i uptake by LLC-PK_1 cells. (A) Na^+-dependent P_i uptake under different sodium concentrations (NaCl was replaced by choline chloride) plus 0.1 mmol/l phosphate. (B) Analysis of sodium-dependency by a plot $1/v$ against $1/[Na]^2$. The K_m for sodium calculated was 56 mmol/l. Each point represents the mean ± S.D. of four determinations.

coefficient of 1.5. A value which indicates that more than one sodium ion parcipitates in the transport event. For the Hill analysis the V_{max} for transport was obtained from a graphical extrapolation of Fig. 3A. This value was similar to that calculated in Fig. 2B. A double reciprocal plot of $1/v$ against $1/Na^2$ of Fig. 3A gave a straight line ($r = 0.988$) as shown in Fig. 3B. This is further evidence in favour of a 2 Na : 1 HPO_4^{2-} stoichiometry. The apparent K_m for sodium at 0.1 mmol/l phosphate was calculated to be 56 mmol/l.

As in a number of other phosphate transport systems [16,17] arsenate was found to inhibit phosphate uptake into LLC-PK_1 cells. Fig. 4 shows the effectiveness of various concentrations of arsenate to inhibit phosphate uptake at a given phosphate

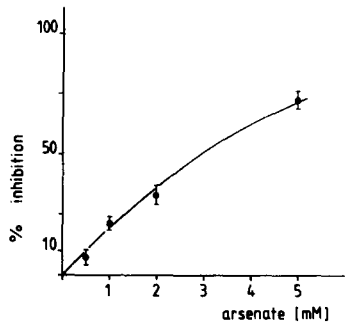

Fig. 4. Inhibition of P_i uptake by LLC-PK_1 cells by arsenate. Sodium-dependent P_i uptake was determined in 137 mmol/l NaCl and 0.1 mmol/l phosphate. Means ± S.D. of four determinations are given.

concentration (80 μmol/l). Over 70% of the uptake is inhibited by 5 mmol/l arsenate. A quantitative analysis of arsenate inhibition of phosphate uptake with a Dixon-Plot (not shown) revealed a complex mechanism of action, and it was not possible to delineate between competitive and non-competitive effects of arsenate upon phosphate uptake. This is not surprising considering that arsenate not only competes with phosphate at a transport site, but also has a diverse range of effects upon cellular metabolism [18], all of which might change driving forces for phosphate uptake.

Modification of phosphate uptake in LLC-PK_1 cells by a number of other inhibitors of phosphate transport is shown in Table II. After a 30 min preincubation with ouabain (1 mmol/l), a manoeuvre to dissipate the sodium gradient by inhibiting active sodium extrusion by the Na-

TABLE II

EFFECTS OF METABOLIC INHIBITORS ON P_i UPTAKE BY LLC-PK_1 CELLS

Additions	P_i uptake [a] (pmol/mg/5 min)	% of control
None	2510 ± 121	
Ouabain, 1 mM [b]	1946 ± 63	78 ($p < 0.01$)
FCCP, 7 μM	782 ± 118	31
Phlorizin, 0.1 mM	2799 ± 84	111

[a] P_i uptake is given as net-sodium dependent P_i-uptake. Each value represents the mean ± S.D. of four determinations.

[b] Cells were preincubated for 30 min at 25°C in the presence of 1 mM ouabain.

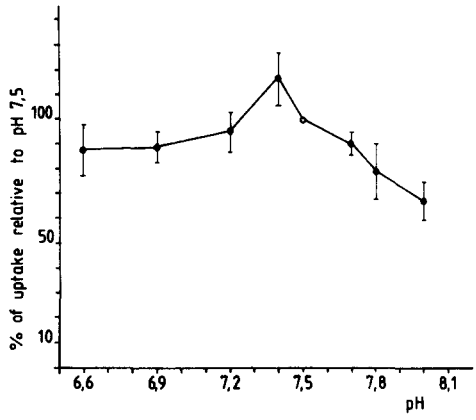

Fig. 5. Dependence of P_i uptake by LLC-PK_1 cells on pH. Sodium-dependent P_i uptake was determined in the presence of 137 mmol/l NaCl and 0.1 mmol/l phosphate at the pH values indicated. Each point represents the mean ± S.D. of four determinations.

pump, resulted in a 22% inhibition in phosphate uptake. A larger inhibition was seen with the proton ionophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), an uncoupler of oxidative metabolism [19]. Phlorizin (0.1 mmol/l), a competitive inhibitor of sodium-dependent D-glucose transport [20], had no significant effect upon phosphate transport by LLC-PK_1, as has been previously reported.

Fig. 5 shows the effect of changing pH of the incubation media over the range of 6.6 to 8.0 upon phosphate uptake. Sodium-dependent phosphate uptake, after an initial decrease from pH 7.4 to 7.2, was independent of pH in the range 6.6 to 7.4. In contrast however, increasing the pH over the range 7.4–8.0 led to a significant decrease in phosphate uptake (35% at pH 8.0). Changing pH had no effect upon the sodium-independent component of uptake.

Discussion

The results presented in this paper clearly demonstrate that LLC-PK_1 pig kidney cells in addition to expressing a Na^+-dependent hexose transporter and a Na^+-dependent amino acid transporter also express a sodium-dependent phosphate transport system. Thus, phosphate uptake into LLC-PK_1 cells is stimulated up to 10-fold by the addition of sodium. Lithium is partially able to substitute for Na^+ but other cation replacements are ineffective. The sodium-dependent component of phosphate uptake conforms to the kinetics of a substrate-saturable process, the apparent K_m for phosphate being 96 ± 15 μmol/l at 137 mmol/l Na. Phosphate uptake measurements made under different sodium concentrations (Fig. 3) suggest that more than one sodium ion is involved in the transport process (Hill coefficient of 1.5) and that the apparent K_m value for the transport process is 56 mmol/l. Phosphate saturation experiments (data not presented) performed under two different sodium concentrations suggest that sodium does not change the affinity of the carrier for phosphate, since the K_m value for phosphate changed only from 98 μmol/l at 137 mmol/l sodium to 82 μmol/l at 50 mmol/l sodium (sodium replaced by choline).

These basic characteristics of phosphate transport by LLC-PK_1 are similar to those reported in abstract form by Rabito [15] and Noronha-Blob et al. [21].

Since LLC-PK_1 cells, originally derived from pig kidney tissue [2] have been shown to exhibit many of the functions characteristic of renal proximal tubular epithelium [1], it may be worthwhile to compare the properties of the sodium-dependent phosphate transport system of LLC-PK_1 cells with what we know of this system in renal tissue. For renal tubular epithelia information on sodium-dependent phosphate transport has been obtained from microperfusion experiments both in vivo and in vitro and from experiments performed upon isolated renal brush-border membrane vesicles [10,22,23]. The phosphate transport mechanism in LLC-PK_1 cells shows many similarities to those described for renal epithelium. The apparent K_m for phosphate for LLC-PK_1, at pH 7.4 and high Na^+, of 96 μmol/l is similar to the K_m previously reported for the transport of phosphate by brush-border membrane vesicles (80–85 μmol/l) [17,24]. Similarly, the apparent K_m for sodium in LLC-PK_1 cells of 56 mmol/l is close to the 60 mmol/l found in renal brush-border membrane vesicles [17].

From both uptake studies with isolated membrane vesicles and from electrophysiological studies [25,26] it has been suggested that at pH 7.4

phosphate is transported by proximal tubule cells in an electroneutral manner using the energy inherent in an inwardly directed sodium gradient. An electroneutral mechanism implies that two sodium ions are transported with one HPO_4^{2-} ion. Although no definite answer can be given at present, indirect evidence (Fig. 3 and Hill coefficient of 1.5) suggests that the coupling between sodium and phosphate in LLC-PK_1 cells may also be 2 : 1. Coupled sodium phosphate transport in LLC-PK_1 cells is also supported by the sensitivity of phosphate uptake to ouabain (Table II).

In contrast to isolated brush-border membrane vesicles [26,27] phosphate uptake into LLC-PK_1 cells is independent of pH over the range 6.6 to 7.2 (Fig. 5). Nevertheless the basic conclusion of that study, that the transport system accepts both monovalent and divalent phosphate ions is still valid for LLC-PK_1 cells. This is shown in Fig. 5, where with a phosphate concentration (100 μmol/l) close to the K_m of the system for phosphate, no significant change in phosphate uptake is observed despite large changes in the ratio of monovalent to divalent phosphate ions (from pH 7.4 to pH 6.3 the divalent form decreases from 80% of the total to only 24%, the pK_2 for phosphate being approximately 6.8). Since this is the case, the decrease in phosphate uptake found above pH 7.5 results not from changes in the systems ability to handle divalent phosphate, but by some other mechanism.

In rat kidney proximal tubular cells, Na^+-dependent phosphate transport is a property of the luminal membrane only, and is not present in basal-lateral membrane vesicles (Hagenbuch, B., personal communication). Since LLC-PK_1 cells retain the same epithelial polarity of cells in vivo it might be expected that the transporter described here is located solely upon the apical cell membrane. At present we can say nothing about the localisation of the carrier; our experiments were performed upon subconfluent cell monolayers (a condition where phosphate has access to both plasma membranes) and also, since LLC-PK_1 cells produce a 'leaky' epithelia [5,6], measurement of phosphate uptake into confluent cell monolayers may also be composed of influx across both cell membranes. Definite location of the transporter to the apical membrane must be made in isolated membrane vesicles, which is the subject of our present research efforts.

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