Study of the mechanism by which the $\text{Na}^+\text{-P}_i$ co-transporter of mouse kidney proximal-tubule cells adjusts to phosphate depletion

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1. Proximal-tubule cells isolated from mouse kidney after digestion with collagenase take up P_i by an Na^+ -dependent and saturable process mediated by the $\text{Na}^+\text{-P}_i$ co-transporter of the brush-border membrane. 2. P_i depletion of the cells is accompanied by a stimulation of P_i -transport activity. Kinetic investigations reveal that V_{max} is increased by 90% and K_m decreased by 50% after P_i depletion. Transport activity returns to normal values after incubation for 30 min at 37 °C of P_i -depleted cells in normal medium containing 1 mM- P_i , but the fall in transport activity under these conditions is inhibited by colchicine. 3. The energy of activation of $\text{Na}^+\text{-P}_i$ co-transport activity of depleted cells differs greatly from that found for normal replete cells. 4. The results provide evidence that stimulation of transport by P_i depletion arises from an increase in the number of carrier sites in the brush-border membrane. Additionally, changes in the properties of the transporter occur which may reflect altered phospholipid-carrier-protein interaction in the P_i -depleted condition.

INTRODUCTION

The activity of the Na^+ -dependent P_i transporter present in the brush-border membrane of the proximal-tubule region of the nephron is important for the homeostatic control of phosphate in man and other animals (Dennis *et al.*, 1979; Gmaj & Murer, 1986; Butterworth, 1987). The activity of the transporter is stimulated when the dietary intake of phosphate is low, and evidence for a change in activity can be obtained within a relatively short time, i.e. by 1 h after the feeding of a low-phosphate meal (Caverzasio & Bonjour, 1985).

Isolated chick proximal-tubule cells (Grahn & Butterworth, 1986) and the kidney epithelial-cell line LLC-PK₁ (Caverzasio *et al.*, 1985) respond to a direct stimulus of low ambient P_i with an increase in Na^+ -dependent P_i uptake. These preparations are believed to provide a model for the transporter of the brush-border membrane of the proximal tubule of the intact kidney, and their stimulation by low P_i therefore suggests that other humoral factors are not involved directly in the process whereby kidney re-absorption of P_i is adjusted to meet the physiological demand for the anion.

The experiments with cultured LLC-PK₁ cells (Caverzasio *et al.*, 1985) suggested that adaptation of transport to low P_i occurs in two stages: a rapid increase that is independent of protein synthesis, and a slower process that may be blocked with inhibitors of protein synthesis. It is possible that rapid adjustment requires the insertion into the brush-border membrane of extra molecules of transporter derived from intracellular stores, or an increase of the intrinsic activity of the existing complement of transporter. These alternatives are not necessarily exclusive, so that an increase in the total number of transport sites may accompany a rise in intrinsic activity. Although some progress is being made in the elucidation of the molecular properties of the P_i transporter (Schali *et al.*, 1986), its identification and quantification in membrane preparations is not yet

possible. Thus evidence for changes in the total number of transport sites and/or a change in the characteristics of the protein can be obtained by indirect methods only. We report here the results of such experiments with an isolated proximal-tubule cell preparation, the aim of which was to determine whether P_i depletion results in an increase in the number of transporter sites.

EXPERIMENTAL

Isolation of mouse kidney proximal-tubule cells

The kidneys were removed from four mice immediately after they were killed, and cut into small pieces with scissors. The divided tissue (approx. 1.5 g) was then transferred to a vial containing 15 ml of Krebs–Ringer bicarbonate medium (Krebs & Henseleit, 1932), containing 10 mM-glucose and 10 mM-pyruvate (isolation medium) plus 24 mg of collagenase and 15 mg of hyaluronidase and which had been warmed to 37 °C. The suspension was stirred continuously and gassed gently with O_2/CO_2 (19:1) and allowed to digest at 37 °C for 25–35 min, depending on the particular batch of collagenase. The suspension was then filtered through nylon mesh to remove large fragments of undigested tissue, and the filtrate was made up to 20 ml by the addition of the isolation medium. The digest was centrifuged at 2000 rev./min for 30 s on an MSE Microcentaur bench centrifuge. The supernatant was discarded and the pellet resuspended in 20 ml of warm isolation medium. Clumps of cells were dispersed by gentle sucking into, and discharge from, a blunt Pasteur pipette. The suspension was centrifuged as before and the pellet washed by resuspension in 20 ml of warm medium, followed by centrifugation. The entire washing step was repeated once more and the final cell preparation suspended in 12 ml of warm oxygenated isolation medium containing 1 mg of bovine serum albumin/ml. The suspension of proximal-tubule cells has a characteristic yellowish

appearance, and light-microscopy shows that the preparation is largely composed of proximal-tubule cells grouped together in clumps of six to eight cells. The kidneys from four mice produce about 15 mg of cell protein.

Microscopy and demonstration of a high alkaline phosphatase activity coupled with an extremely low hexokinase activity indicated that the preparation was 90–95% proximal-tubule material contaminated with about 5% of material originating from the distal and collecting tubules. Provided that the cells are kept at 37 °C under an atmosphere of O₂/CO₂, they remain viable for 45–60 min, as judged by 98% exclusion of Trypan Blue, by measurement of coupled respiration, by a low leakage of lactate dehydrogenase and by gluconeogenic activity.

Phosphate-depleted cells

The same isolation procedure was followed, except that the medium was devoid of P_i during the collagenase digestion and also the washing steps (a total period of 45 min). The cells were finally suspended in P_i-free isolation medium.

Measurement of P_i uptake

The method was essentially the same as that described by Grahn *et al.* (1985) with chick kidney cells. The tubule cells were incubated at 37 °C with gentle shaking in isolation medium containing 1 mM-P_i and 1 mg of bovine serum albumin/ml. The reaction mixtures of 3 ml volume (with approx. 2.5 mg of cell protein/ml) were contained in glass universal containers capped with Parafilm. The space above the liquid was filled with O₂/CO₂ (19:1). At zero time, carrier-free [³²P]P_i was added to a concentration of 2 µCi/ml. When measuring uptake by P_i-depleted cells, sufficient anion was added to bring the concentration in the suspension to 1 mM, followed by [³²P]P_i 20 s later. Timing was begun from this point. Triplicate samples (0.2 ml) were removed at 1 min intervals up to 4 min and transferred to Microfuge tubes containing 0.4 ml of silicone oil/dinonyl phthalate (3:2, v/v) layered over 0.05 ml of 3 M-HClO₄. The tubes were centrifuged for 1 min in a Microfuge, after which the upper aqueous layer was removed by aspiration. The tubes were sliced at the level of the oil layer with a hot blade, and the HClO₄/cell layer was dropped into scintillation fluid for determination of radioactivity. The initial rate of P_i uptake was calculated from a computer-generated linear plot of accumulated P_i against time.

For measurements of uptake at different P_i concentrations, suspensions of normal cells (in 1 mM-P_i) were microcentrifuged and washed twice by suspension in P_i-free medium, followed by centrifugation. The cells were resuspended finally in the P_i-free medium. The total washing procedure was completed in approx. 5 min. P_i was added to portions of suspension to provide the required range of concentrations, followed by the radioactive tracer 20 s later. The procedure for P_i-depleted cells was similar, except that the preliminary microcentrifugation and washing steps were not needed.

When the effect on the rate of P_i uptake of maintaining the cells in 1 mM-P_i was being tested, portions of cells suspended in normal medium were incubated at 37 °C. At a suitable time, tracer was added to a sample and the

Fig. 1. Time-dependence of P_i uptake into cells in the presence of an Na⁺ gradient and 1 mM-P_i.

The results are taken from a single experiment conducted with P_i-depleted cells. The data points are means of triplicate assays made at each time point and have been corrected for non-internalized P_i, i.e. that binding to the membrane at zero time, which amounted to 0.5 nmol/mg of protein.

rate of uptake determined by the methods described above.

For experiments where different experimental protocols were required, the appropriate information is provided in the Results section.

Other methods and materials

Hexokinase was determined by the method of Chou & Wilson (1975), lactate dehydrogenase by the method of Vassault (1983) and alkaline phosphatase by the procedure of Jahan & Butterworth (1986). Gluconeogenesis from pyruvate was measured by the method described by Grahn *et al.* (1985). Protein was determined by the biuret method, with bovine serum albumin as standard (Plummer, 1987).

Silicone oil AR 200 was obtained from Wacker Chemie A.G., Walton-on-Thames, Surrey, U.K. Collagenase, hyaluronidase and the enzymes required for hexokinase and glucose determinations were from Sigma, Poole, Dorset, U.K. Scinttran liquid-scintillation cocktail T was purchased from BDH, Poole, Dorset, U.K.

RESULTS

Mouse kidney proximal-tubule cells take up P_i by an Na⁺-dependent process. Replacement of Na⁺ ions in the isolation medium with an equal concentration of choline strongly inhibits P_i uptake. In this respect the cells resemble isolated chick kidney tubular cells (Grahn & Butterworth, 1981), and this provides evidence that the measured uptake is largely mediated by the Na⁺-P_i co-transporter and therefore represents transport across the brush-border membrane of the tubule cells. The uptake was linear with time of incubation for at least 5 min (Fig. 1), but the relationship between the rate of P_i accumulation and the concentration of cells in the incubation medium deviated from linearity at concentrations above

Fig. 2. Concentration-dependence of the rate of P_i uptake

●, Uptake by normal P_i -replete cells in the presence of NaCl; ○, uptake by P_i -depleted cells in the presence of NaCl; □, uptake by both normal and depleted cells when NaCl was replaced by choline chloride. For Na^+ -supported uptake, each point is the mean of three separate experiments. Uptake in the presence of choline was identical for both types of cell, i.e. the points are means of six separate experiments.

3 mg/ml. For this reason, uptake experiments were always performed with incubation mixtures containing 2.5 mg of cell protein/ml.

A quantity of P_i is accumulated instantaneously by the cells when they are mixed at 0 °C with various concentrations of P_i . This 'instant' labelling of cells presumably represents binding of P_i to the plasma membrane, i.e. the P_i is not internalized. The amount of bound P_i was a linear function of the concentration of ambient P_i , and no evidence of saturation was obtained with concentrations up to 5 mM- P_i .

The instant-binding results contrasted with those shown in Fig. 2, which were obtained at 37 °C and where the rate of P_i uptake was computed from the increase of labelling over a period of 4 min. Fig. 2 shows P_i uptake as a function of concentration, into normal P_i -replete cells and into P_i -depleted cells, and also uptake under conditions in which NaCl was replaced by choline chloride. It is clear (a) that uptake in the presence of NaCl is saturable and (b) that P_i depletion stimulates the rate of uptake at all measured P_i concentrations. Replotting the data for the Na^+ -dependent fraction of uptake by a linear transformation of the Michaelis-Menten equation (Fig. 3) shows that P_i depletion affects the kinetics of transport. Kinetic parameters and their standard errors were calculated from the data shown in Fig. 3 (based on three separate experiments with both normal and depleted cells) plus data obtained from two further separate experiments performed with normal and depleted cells. V_{max} is 0.40 ± 0.02 and 0.767 ± 0.111 nmol/min per mg for normal and depleted cells respectively (significantly different at $P < 0.01$), whereas the equivalent K_m values are 1.05 ± 0.09 and 0.447 ± 0.124 mM (significantly different at $P < 0.05$). The significance was tested by the method suggested by Cleland (1967).

Fig. 3. Kinetics of Na^+ -dependent uptake

Double-reciprocal plots of the data from Fig. 2. The uptake rate at each $[P_i]$ was corrected for diffusional entry by subtraction of the rate obtained with choline chloride. ●, Normal cells; ○, depleted cells. Kinetic parameters reported in the text were calculated by weighted linear regression (Wilkinson, 1961).

Fig. 4. Changes in the rate of Na^+ -dependent uptake after incubation of the cells in isolation medium at 37 °C

○, Normal P_i -replete cells; △, P_i -depleted cells; ▲, P_i -depleted cells incubated in the presence of colchicine (16 µg/ml). Each point is the mean of three separate experiments. The bars indicate the ranges of the experimental values.

When cells that have been depleted of P_i are subsequently incubated in the normal isolation medium containing 1 mM- P_i , the rate of Na^+ -dependent uptake decreases with time of incubation (Fig. 4). Thus repletion with P_i returns the activity of the transport system to the basal value. By 30 min the activity had fallen to a value that was close to that of unstimulated cells. Studies were

Fig. 5. Arrhenius plot of Na^+ -dependent P_i uptake

●, Normal P_i -replete cells; ○, P_i -depleted cells. The plots present grouped data obtained from three separate experiments performed with both normal and P_i -depleted cells.

not continued beyond 30 min, because of fears that the viability of the cells would become a limiting factor. Fig. 4 shows, however, that the transport potential of the cells seems to remain intact for 30 min, because the Na^+ -dependent P_i uptake of normal P_i -replete cells remained constant throughout the 30 min period.

When the cytoskeletal-disruptive agent colchicine was included in the incubation mixtures at $16 \mu\text{g/ml}$, the time-dependent fall in the transport activity of P_i -depleted cells was largely prevented. Higher concentrations of colchicine were found to be cytotoxic, and concentrations lower than $16 \mu\text{g/ml}$ were relatively ineffective.

P_i transport by both replete and P_i -depleted cells was studied at several temperatures between 14 and 37°C . In these experiments it was necessary to maintain the cells at 37°C until just before transport measurements, to ensure that the Na^+ gradient across the cell membrane was not disturbed. Thus cells were cooled to the required temperature for the uptake experiments by a carefully controlled immersion in ice-cold water for up to 30 s. Uptake was monitored for 3 min rather than for the usual 4 min period, to minimize difficulties arising from the dissipation of the Na^+ gradient during the actual experiment. For both normal and depleted cells, $1 \text{ mM-}\text{P}_i$ was present during the temperature-equilibration period, and then the radiolabel was added at zero time.

The results of the temperature studies are shown in Fig. 5, where data are plotted in the form of Arrhenius plots of $\log v$ against $1/T$. The plots were linear, and within the limits of experimental error exhibited no obvious discontinuities. Depletion of the cells of P_i increased the slope of the Arrhenius plots, so that the calculated energies of activation were 28 kJ/mol and 65 kJ/mol for normal and depleted cells respectively. A surprising feature of the results was that depletion, which is associated with an increased transport activity, has a higher activation energy.

DISCUSSION

The Na^+ -dependent P_i transporter of mouse proximal-tubule cells responds to a depletion episode with an increase in activity. Both the V_{max} and the K_m of transport appear to be affected in directions that should increase the efficiency of P_i uptake from the luminal fluid of the kidney proximal tubule, i.e. V_{max} is raised but K_m is decreased. Previous reports both from this laboratory and from others (see the Introduction) usually claim that the change in kinetic parameters affects V_{max} only. The discrepancy may arise from the imprecision of transport K_m calculations. A doubling of V_{max} can be recognized unequivocally, but a less than 4-fold change in K_m is difficult to establish reliably. Of interest for our work is whether the change in kinetic parameters reflects a change in the intrinsic activity of the transporter accompanying P_i depletion, or whether the number of transport sites has increased.

It could be argued that part of the increased rate of uptake into depleted cells arises from a more favourable electrochemical gradient for the entry of P_i , because of a lower cytosolic concentration of the anion. Diffusional uptake, measured in the absence of Na^+ , occurs at an identical rate in normal and depleted cells, however, suggesting that the cells differ little with respect to cytosolic P_i concentration. Indeed, when measurements have been made of cytosolic P_i in a number of cell types, it has been found that the anion concentration is not greatly influenced by the concentration of P_i in the surrounding medium (Bevington *et al.*, 1986; Grahn & Butterworth, 1986). It seems that cytosolic P_i is buffered at the expense of organic phosphate and/or mitochondrial P_i , perhaps. The experiments in which the effects of colchicine were tested show that the initial rate of uptake remains high and constant for depleted and normal cells throughout the period of incubation in $1 \text{ mM-}\text{P}_i$. This finding fits a conclusion that the increased entry rate into depleted cells results from an increased transport capacity rather than from a more favourable electrochemical gradient for P_i accumulation.

By studying the temperature-dependence of the transporter, it was hoped that it might be possible to ascertain whether the intrinsic properties are altered during depletion. A measured energy of activation (E) should largely reflect the rate constant for the rate-limiting step in transport (k_t), so that a change in the character of the transporter should affect k_t and therefore the energy of activation. Unfortunately, interpretation of the experiments has proved difficult. P_i depletion increases E , which is suggestive of an altered k_t and thus a modified transporter. The temperature-dependence of the P_i -transport activity of brush-border membrane vesicles obtained from rat kidney has been studied (Brunette & Beliveau, 1984), with the finding, however, that a break appears in the Arrhenius plot at 23°C . The break was interpreted as signifying a change in the physical state of the membrane. Le Grimmelc *et al.* (1982) reported a break at 26°C in the Arrhenius plot of alkaline phosphatase activity in kidney brush-border membrane, which was assumed to represent a change in membrane fluidity. Calculation of E from the transport data given by Brunette & Beliveau (1984) gives approximate values of 100 kJ/mol and 13 kJ/mol below and above the transition point (23°C) respectively. These values are of similar order to the differences that we find between the

normal and P_i -depleted cells. It is possible that cellular depletion of P_i is accompanied by metabolic effects which influence the composition and physical properties of the membrane, and that alterations of transport activity are not therefore a direct property of the transporter itself, but arise from changes in interactions between the carrier protein and surrounding phospholipid molecules. Investigation of the phospholipid composition of normal and depleted cells may aid in providing an answer to this question.

Colchicine hinders the fall in activity of P_i transport when P_i -depleted cells are returned to medium containing physiological concentrations of P_i . The agent is expected to interfere with membrane traffic, and so the result can be interpreted as evidence that molecules of transporter can be transferred from the membrane to an internal store as the cell senses normal P_i concentrations in the bathing medium. It therefore seems highly likely that the increase in transport activity accompanying P_i depletion does involve a change in the number of transport sites in the membrane.

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