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Electrogenicity of phosphate transport by renal brush-border membranes

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Phosphate uptake by rat renal brush-border membrane vesicles was studied under experimental conditions where transmembrane electrical potential ($\Delta\psi$) could be manipulated. Experiments were performed under initial rate conditions to avoid complications associated with the dissipation of ion gradients. First, phosphate uptake was shown to be strongly affected by the nature of Na^+ co-anions, the highest rates of uptake being observed with 100 mM-NaSCN (1.010 ± 0.086 pmol/5 s per μg of protein) and the lowest with 50 mM-Na_2SO_4 (0.331 ± 0.046 pmol/5 s per μg of protein). Anion substitution studies showed that potency of the effect of the co-anions was in the order thiocyanate > nitrate > chloride > isethionate > gluconate > sulphate, which correlates with the known permeability of the membrane to these anions and thus to the generation of transmembrane electrical potentials of decreasing magnitude (inside negative). The stimulation by ion-diffusion-induced potential was observed from pH 6.5 to 8.5, indicating that the transport of both monovalent and divalent phosphate was affected. In addition, inside-negative membrane potentials were generated by valinomycin-induced diffusion of K^+ from K^+-loaded vesicles and showed a 57% stimulation of phosphate uptake, at pH 7.5. Similar experiments with H^+-loaded vesicles, in the presence of carbonyl cyanide *m*-chlorophenylhydrazone gave a 50% stimulation compared with controls. Inside-positive membrane potentials were also induced by reversal of the K^+ gradient (outside > inside) in the presence of valinomycin and gave 58% inhibition of phosphate uptake. The membrane-potential dependency of phosphate uptake was finally analysed under thermodynamic equilibrium, and a stimulation by inside-negative potential was observed. The transport of phosphate was thus driven against a concentration gradient by a membrane potential, implicating the net transfer of a positive charge during the translocation process. These results indicate a major contribution of electrical potential to phosphate uptake in renal brush-border membranes.

INTRODUCTION

Reabsorption of phosphate in the kidney takes place predominantly in the proximal tubule (Dennis *et al.*, 1979). The metabolic regulation includes many effectors such as vitamin D, parathyroid hormone, insulin, diet, acid/base status, Ca^{2+} and NAD^+–NADH (Dousa & Kempson, 1982). With the introduction of membrane vesicles, which allow a better control of experimental parameters (Crane, 1977), our understanding of the mechanisms by which reabsorption occurs has greatly increased. Sodium-dependent phosphate uptake into kidney brush-border membrane vesicles has been characterized (Hoffmann *et al.*, 1976; Cheng & Sacktor, 1981; Murer & Burckhardt, 1983; Hammerman & Schwab, 1984; Bindels *et al.*, 1987). The sodium gradient was found to induce conformational changes in the carrier molecule (Béliveau & Strevey, 1987).

The nature of the transported phosphate anion, either monovalent or divalent, remains controversial, as does the pH dependency of the transport system *per se*. An increased rate of phosphate transport was found by increasing the pH of the incubation medium (Burckhardt *et al.*, 1981; Cheng & Sacktor, 1981; Brunette *et al.*, 1984), thus suggesting that the divalent anion is the preferentially transported substrate. The pH dependency of phosphate transport is, however, strongly influenced by the sodium concentration, an observation that led to the suggestion that the major effect of pH is related to the interaction of the transporter with sodium, rather than to a preferential transport of the divalent phosphate anion (Amstutz *et al.*, 1985). In intestinal brush-border membrane vesicles, the pH-dependency of phosphate transport appears to reflect intrinsic properties of the transport mechanism and has been related to changes in the affinity for sodium (Danisi *et al.*, 1984). Moreover, intestinal phosphate transport is electrogenic at either acid or alkaline pH.

Previous results with vesicles isolated from proximal tubules of rabbit kidney, seem to imply an electroneutral phosphate transport (Cheng & Sacktor, 1981), but a small electrogenic component has been detected in rat vesicles (Hoffmann *et al.*, 1976). Electrophysiological studies have shown similar but not identical ionic conductivities in brush border membrane vesicles from both tissues (Wright, 1984). Renal vesicles show high conductance pathways for H^+, Na^+ and K^+ (Burnham *et al.*, 1982) and it is thus possible that a rapid dissipation

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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of the ion gradients gives rise to early collapse of the driving force of the transmembrane electrical potentials and precludes the observation of any effect of electrical potential on phosphate uptake. In the present study, membrane vesicles from rat kidney were used to study the influence of electrical potentials on phosphate uptake in initial rate conditions. Our results show a strong dependency of phosphate transport on transmembrane diffusion potentials generated by either anion substitution or ionophores. Experiments performed in zero-*trans* or in thermodynamic equilibrium conditions both show that phosphate uptake is electrogenic and modulated by membrane electrical potential.

MATERIALS AND METHODS

Preparation of brush-border membranes from rat kidney cortex

Brush-border membranes were prepared as described by Booth & Kenny (1974). The purified brush-border membrane vesicles were resuspended to a final protein concentration of 10 mg/ml in 10 mM-Tris/Hepes buffer (pH 7.5) containing 300 mM-mannitol. The enrichments in alkaline phosphatase and $Na^+ + K^+$-dependent ATPase were 14.2 ± 0.3- and 0.41 ± 0.1-fold (means ± S.E.M. of five experiments) respectively.

Uptake studies

The rapid filtration technique of Hopfer *et al.* (1973) was used for uptake studies. Incubation media contained in a 30 μl final volume: 20 mM-Tris/Hepes buffer (pH 7.5)/100 mM-mannitol/0.2 mM-$^{32}P_i$ and 100 mM of the given sodium salt. Uptake studies, performed at least in triplicate at 25 °C, were initiated by the addition of 60–100 μg of protein. After incubation, the reactions were stopped by the addition of 1 ml of ice-cold stop solution consisting of 5 mM-Tris/Hepes buffer (pH 7.5)/300 mM-mannitol/10 mM-Na_3AsO_4. The suspension was applied to a 0.45 μm pore-size Millipore filter under vacuum. Filters were washed with 8 ml of ice-cold stop solution and then processed for liquid scintillation counting. Uptake values were corrected for non-specific absorption by vesicles and filters in a separate assay where vesicles and incubation medium were added directly to the stop solution. Non-specific binding never exceeded 8.3% of normal uptake values. Na^+-dependent uptake was calculated as the difference betwen uptake values in Na^+-anion and K^+-anion conditions. When not indicated, standard errors were less than 8% of the mean. Glucose uptake was used as a routine control for induction of electrical potentials in the different experimental conditions presented (Kessler & Semenza, 1983). Statistical analyses were made using the Student *t*-test.

Valinomycin and CCCP were obtained from Sigma (St. Louis, MO, U.S.A.) and $^{32}P_i$ (carrier-free) was from New England Nuclear (Boston, MA, U.S.A.). Other chemicals were of the highest purity commercially available.

RESULTS

Anion-dependence of phosphate entry

Incubation of vesicles with anions of different permeabilities results in different stimulation of phosphate uptake. Fig. 1 shows the time course of phosphate uptake in the presence of a 100 mM-Na^+ gradient with

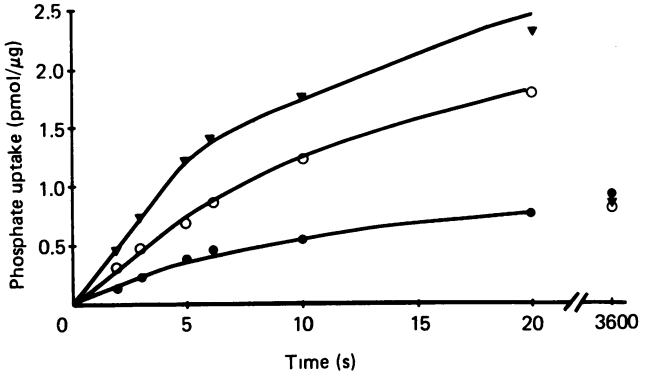

Fig. 1. Influence of co-anions on time course of phosphate uptake

Vesicles resuspended in 300 mM-mannitol/20 mM-Tris/Hepes (pH 7.5) were incubated for indicated times in 100 mM-mannitol/20 mM-Tris/Hepes (pH 7.5)/0.2 mM-$^{32}P_i$ and 100 mM of either NaSCN (▼), NaCl (○) or sodium gluconate (●).

different co-anions. Replacement of chloride by thiocyanate, which permeates more easily across the membrane, gave rise to a stimulation of phosphate uptake in the initial period. Replacement of chloride by gluconate, a much less permeant anion, caused a decrease in uptake. When thermodynamic equilibrium was reached, the intravesicular contents of substrate were not affected by anion substitution, as indicated by the identical values at 60 min. Equilibrium uptake of phosphate has already been shown to consist of two compartments: the intravesicular volume and a small, non-specific binding component (Béliveau & Strevey, 1988) which is easily corrected for because it represents approx. 10% of the total radioactivity. Measurement of intravesicular volume gave a value of 1.7 μl/mg of protein, identical with the volume obtained with D-glucose for which there is no binding. Moreover, anion substitution caused no variations in D-glucose equilibrium values. The effect observed in initial time is probably not due to variations in the intravesicular volume with the different anions unless we suppose a transitory variation in vesicle size. Phosphate uptake was also studied with other anions of different permeabilities. The results shown in Table 1 indicate that nitrate had a stimulating effect but isethionate and sulphate had an inhibitory effect on phosphate uptake, compared with uptake measured in the presence of chloride at initial time. The potency order of the effect of Na^+ co-anion on uptake is thus thiocyanate > nitrate > chloride > isethionate > gluconate > sulphate.

In order to show that the differential effects of anions on phosphate entry were not due to a direct interaction of those anions with the carrier, experiments were conducted with an equal amount of the anions on both sides of the membrane. Under these conditions, the carrier still faces a 100 mM-Na^+ gradient, but the membrane diffusion potential induced by anion translocation is much reduced. Table 1 shows that, under such conditions, phosphate entry was similar in the presence of either sulphate or nitrate ions. This is greatly different from the situation in which an anion gradient was present and where the nitrate gradient induced a

Table 1. Effect of ionic composition on phosphate uptake

Membrane vesicles were pre-incubated in 20 mM-Tris/Hepes (pH 7.5) and 300 mM-mannitol, or 100 mM-mannitol and 100 mM-KNO_3, or 100 mM-mannitol and 50 mM-K_2SO_4. They were then incubated in 100 mM-mannitol/20 mM-Tris/Hepes (pH 7.5)/0.2 mM-$^{32}P_i$ and 100 mM of either NaSCN, $NaNO_3$, NaCl, sodium isethionate, sodium gluconate or 50 mM-Na_2SO_4.

Ionic composition (outside/inside)	Phosphate uptake (pmol/5 s per μg of protein)	(%)	(pmol/60 min per μg of protein)
NaSCN/mannitol	1.01±0.086	100	0.804±0.062
$NaNO_3$/mannitol	0.952±0.064	94	0.756±0.084
NaCl/mannitol	0.841±0.068	83	0.881±0.051
Sodium isethionate/mannitol	0.521±0.084	51	0.794±0.082
Sodium gluconate/mannitol	0.496±0.052	49	0.765±0.081
Na_2SO_4/mannitol	0.331±0.046	33	0.834±0.073
$NaNO_3/KNO_3$	0.495±0.097	49	0.801±0.047
Na_2SO_4/K_2SO_4	0.446±0.064	44	0.796±0.081

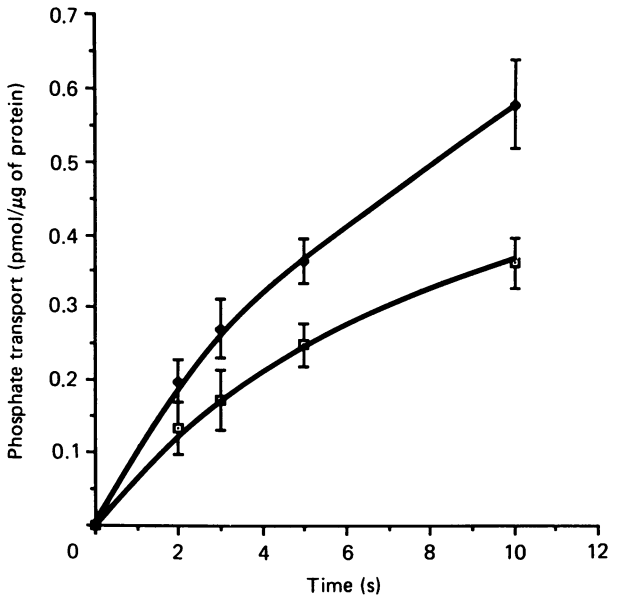

Fig. 2. Effect of an inside-negative potential induced by valinomycin on phosphate transport at pH 7.5

Vesicles were preincubated in 100 mM-mannitol/100 mM-potassium gluconate/20 mM-Tris/Hepes, pH 7.5. They were then incubated in 100 mM-sodium gluconate/20 mM-Tris/Hepes (pH 7.5)/0.2 mM-$^{32}P_i$ in the presence (●) or absence (□) of valinomycin (10 μg/mg of protein). Controls were run in 2% with ethanol, the valinomycin solvent.

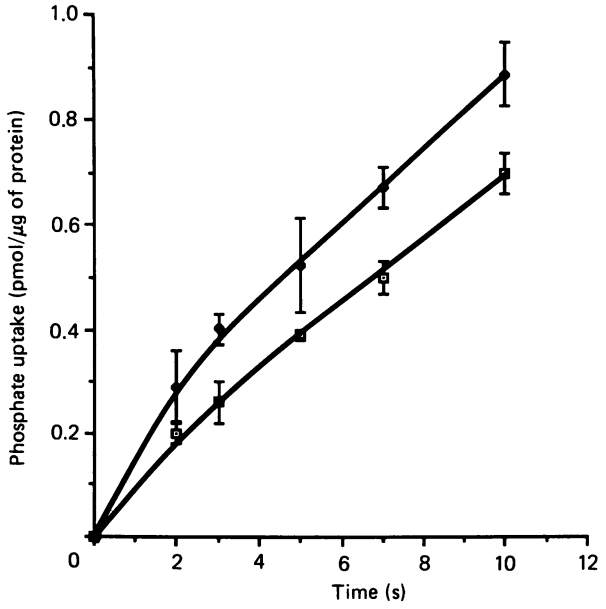

Fig. 3. Effect of a negative inside potential induced by CCCP on phosphate entry

Vesicles were preincubated in 250 mM-mannitol/50 mM-Mes/Tris, pH 5.5. They were incubated in 100 mM-mannitol/100 mM-sodium gluconate/20 mM-Tris/Hepes (pH 7.5)/0.2 mM-$^{32}P_i$ in the presence (●) or absence (□) of CCCP (10 μM). Controls were made with ethanol, the CCCP solvent.

stimulation of phosphate uptake 2.8-times higher than the sulphate gradient (0.952±0.096 pmol/5 s per μg of protein compared with 0.331±0.039 pmol/5 s per μg of protein). Reducing the electrical potential thus neutralizes the anion effect, indicating that the observed differences are not due to competition between anions and phosphate on the *cis* side.

Phosphate entry under the influence of membrane potential induced by ionophores

Phosphate uptake was studied under conditions where a transitory transmembrane electrical potential was generated by the use of ionophores to induce the diffusion of cations. Fig. 2 shows the results of experiments where inside electronegative potentials were induced by the addition of valinomycin to K^+ gluconate-loaded vesicles. The brush-border membrane shows low permeability for gluconate, in contrast with the high permeability for K^+ induced by valinomycin. The induced exit of K^+ down its electrochemical gradient generates a membrane potential (negative inside). Transport of D-glucose entry has thus been shown to be greatly sensitive to potentials induced by valinomycin (Kessler & Semenza, 1983). D-glucose uptake was used to verify the formation of membrane potentials. Phosphate entry, studied under the influence of an inside negative membrane potential,

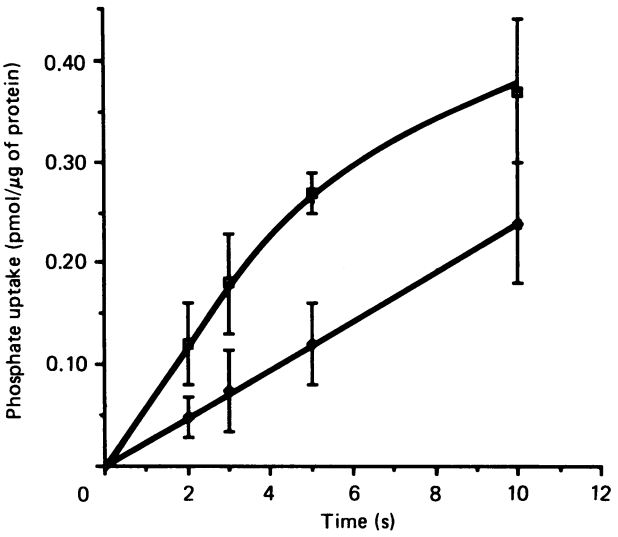

Fig. 4. Effect of a positive inside potential on phosphate entry

Vesicles were resuspended in 300 mM-mannitol/20 mM-Tris/Hepes, pH 7.5. Incubation was made in 50 mM-potassium gluconate/100 mM-sodium gluconate/20 mM-Tris/Hepes (pH 7.5)/0.2 mM-$^{32}P_i$ in the presence (●) or absence (□) of valinomycin (10 μg/mg of protein).

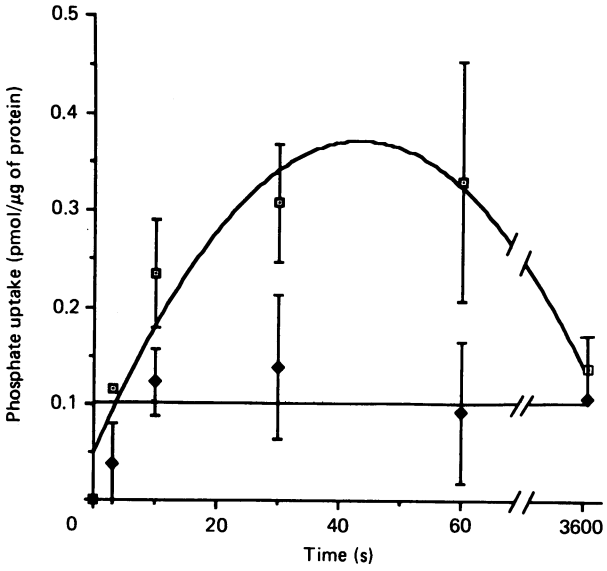

Fig. 5. Thermodynamic test for electrogenicity of phosphate entry

Membrane vesicles were pre-incubated in 100 mM-sodium gluconate/100 mM-mannitol/0.2 mM-$^{32}P_i$/20 mM-Hepes/Tris, pH 7.5. They were then incubated in 100 mM-mannitol/0.2 mM-$^{32}P_i$/20 mM-Hepes/Tris (pH 7.5)/100 mM-sodium nitrate (□) or 100 mM-sodium gluconate (◆).

was stimulated by valinomycin (Fig. 2), when measured in initial rate conditions. After longer times of incubation, the stimulatory effect was lost progressively, presumably because of a dissipation of the induced potential. Phosphate uptake at equilibrium was unaltered by valinomycin as compared with the ethanol control, indicating identical intravesicular volumes in both conditions (not shown). In fact, phosphate uptake was insensitive to ethanol concentrations up to 4%, indicating a high resistance of brush-border membrane vesicles towards this solvent.

Inside-negative potentials can also be induced by proton diffusion from acid-loaded vesicles in the presence of CCCP, a proton ionophore. Membrane vesicles were loaded at pH 5.5, and phosphate entry was measured in the presence of CCCP (Fig. 3). The high permeability to H^+ induced by the ionophore causes a large exit of protons and consequently the generation of an inside-negative potential. CCCP caused stimulation of phosphate at the initial times of incubation. However, once again, the stimulatory effect disappeared as a function of incubation time. Equilibrium values were identical for CCCP and ethanol-treated vesicles, indicating no specific action of CCCP itself on the vesicles. CCCP and valinomycin were also found to be without any effect in the absence of H^+ or K^+ gradients. These phenomena were observed only in the presence of imposed gradients, indicating that it is the ionophoric properties of these molecules that are responsible for the observed effects.

To test further the electrogenicity of phosphate transport, experiments were performed in which the membrane potential was reversed. An inward potassium gradient was found to cause an important inhibition (58%) of phosphate entry in the presence of valinomycin (Fig. 4). A proton gradient (outside > inside) could not be used because the Na^+-dependent uptake of phosphate was too small at pH 5.5. It is thus clear that the presence of potassium or protons themselves inside or outside the vesicles cannot be responsible for the observed effects. The reversibility of the effects reported here is an indication of direct involvement of the transmembrane electrical potential.

Table 2. Effect of pH on phosphate uptake

Membrane vesicles were incubated in 100 mM-mannitol/100 mM-sodium gluconate or sodium nitrate/0.2 mM-$^{32}P_i$ and 20 mM of either Mes/Tris (pH 6.5), Hepes/Tris (pH 7.5) or Tris/HCl (pH 8.5).

pH	Incubation medium + ...	Phosphate uptake (pmol/5 s per μg of protein) Sodium gluconate	Sodium nitrate
6.5		0.185 ± 0.024	0.428 ± 0.031
7.5		0.452 ± 0.059	0.840 ± 0.014
8.5		0.675 ± 0.010	0.885 ± 0.036

Electrogenicity in the absence of substrate concentration gradients

A test for electrogenicity of phosphate transport is presented in Fig. 5. Here, membrane vesicles were pre-equilibrated in a buffer containing sodium gluconate, mannitol, and [^{32}P]phosphate. They were then transferred into a medium containing $NaNO_3$, mannitol, phosphate, and [^{32}P]phosphate. In these conditions the sodium phosphate carrier operates in the absence of a substrate concentration gradient. The induction of a potential (inside negative) by nitrate diffusion led to hyperaccumu-

lation of phosphate. A control experiment performed by substituting gluconate for nitrate in the incubation medium showed no uptake over the equilibrium value, indicating that it is the difference between the high permeability of the membrane towards nitrate that is responsible for the induction of the electrical potential. This hyperaccumulation of phosphate was transitory and disappeared after the equilibration of nitrate and gluconate which corresponded to the dissipation of the electrical potential. These results constitute strong thermodynamic evidence for charge translocation associated with the sodium-dependent phosphate entry. The results presented here have been corrected for the sodium-independent phosphate entry by similar experiments in which sodium co-anions were replaced by potassium co-anions.

pH dependency of membrane potential effect

The effect of pH was studied by incubating the vesicles in media containing nitrate or gluconate at different pH values. The strong dependence of phosphate transport upon pH is obvious: compared with transport measured at pH 7.5 where the divalent phosphate is dominant, transport at pH 6.5 is only 41% in the presence of gluconate or 51% in the presence of nitrate, which is in agreement with a preferential transport of divalent phosphate, as already reported (Cheng & Sacktor, 1981; Brunette *et al.*, 1984; Amstutz *et al.*, 1985). As shown in Table 2, the stimulation by nitrate over gluconate was observed at all pH values tested, i.e. stimulation of 231, 185 and 131% at pH 6.5, 7.5 and 8.5, respectively. Thus the negative-inside potential generated by the elevated diffusion of nitrate was effective in conditions where the monovalent form is dominant ($HPO_4^{2-}/H_2PO_4^- = 0.50$ at pH 6.5) and in conditions where the divalent form is largely in excess ($HPO_4^{2-}/H_2PO_4^- = 50$ at pH 8.5).

DISCUSSION

Phosphate transport has been reported to be strongly dependent upon the presence of sodium in the incubation medium (Hoffmann *et al.*, 1976; Cheng & Sacktor, 1981). The results presented here indicate that phosphate transport across the luminal membrane is also strongly dependent upon the presence of a transmembrane electrical potential. The influence of Na^+ co-anions on phosphate entry indicates that the process is sensitive to inside-negative potentials and thus associated with the translocation of a positive charge. This is confirmed by the fact that inside negative potentials induced either by valinomycin or CCCP have the same stimulatory effect. The reverse experiments, in which an inside positive potential is induced by valinomycin, has the opposite effect. These two sets of experiments indicate that the observed effects are not artefacts due either to non-specific interactions of the ionophores with the transport process or to the different ionic media used in these experiments. The behaviour of the carrier is exactly as would be predicted by a rheogenic model.

In conditions where the carrier faces equilibrium concentrations of its two substrates, on the *cis* and on the *trans* sides, the generation of a negative inside membrane potential is still able to stimulate phosphate entry. Electrical energy is thus transduced by the phosphate carrier into a chemical energy, in the form of a phosphate gradient. Transient accumulation of phosphate above equilibrium values was made possible by an inside negative potential in the absence of a sodium gradient. The transport of phosphate is thus driven against a concentration gradient by a membrane potential, implicating the net transfer of a positive charge during the translocation process.

If the chemical coupling of phosphate entry with sodium is a well-established fact for the brush-border membrane, the electrical coupling remains much more controversial. Hoffmann *et al.* (1976) found a small electrogenic effect in rat kidney vesicles. Their conclusion was that the divalent form was transported in an electroneutral manner with two Na^+ ions, but that monovalent phosphate entered the cell as a positively charged complex. Our results show that the transport of divalent as well as monovalent phosphate is modulated by this potential. These results suggest that the modulation of phosphate transport by the membrane potential is a direct consequence of the molecular properties of the carrier itself rather than an indirect effect of this potential for the preferential transport of one ionic form of phosphate. Cheng & Sacktor (1981) made an extensive study of phosphate transport and their conclusion was electroneutrality, with variable stoichiometries for the carrier, depending upon the phosphate species translocated. The reasons for the discrepancy between these data and our own are not clear. Firstly, the species difference (rat and rabbit) can be noted, as it is already known that phosphate transport differs in many aspects between both species, including their transport capacity; $V_{max.}$ is 4 times higher in the rat than in the rabbit (Béliveau & Brunette, 1984). Secondly, the vesicles were prepared differently; rabbit kidney vesicles (Cheng & Sacktor, 1981) were prepared with a Ca^{2+} precipitation method and rat vesicles with Mg^{2+} precipitation. The K^+ permeabilities of the Ca^{2+}-prepared vesicles are 50–120% higher than in vesicles prepared by the Mg^{2+} precipitation method (Sabolic & Burckhardt, 1984). H^+ permeability is also 50–75% higher. It is thus possible that higher permeabilities of the membrane lead to faster dissipation of the ionic gradients. The efficiency of membrane diffusion potentials induced by ionophores in supporting stimulation of glucose uptake in rabbit vesicles could be simply due to the lower magnitude of electrical potentials needed for glucose uptake as compared with those required for phosphate uptake. Thirdly the uptake was measured at different times; 5 s for rat and 20 s for rabbit vesicles. In rat vesicles, uptake is not linear after 7 s and therefore measurements taken after longer periods do not correspond to initial rate conditions. It is thus probable that the electrical gradient induced by the ionophores or by anion substitution would change with time and alter the properties of the transport system. As already pointed out, the importance of measuring the entry in initial rate conditions is crucial in order to avoid artefacts due to dissipation of the induced gradients.

The implication of electrical potential in energizing phosphate transport is suggested by the fact that glucose and alanine transport, which contribute to the transepithelial electrical potential difference, inhibit phosphate reabsorption (Dennis & Brazy, 1978), presumably by depolarizing the luminal membrane. Bicarbonate reabsorption, which is not linked to alteration of the electrical potential difference (Burg & Green, 1977), does

not alter phosphate reabsorption (Dennis & Brazy, 1978). The fact that phlorizin decreases sodium transport while stimulating phosphate reabsorption (Dennis & Brazy, 1978) is another indication that both electrical and chemical coupling are implicated in the transmembrane translocation of phosphate. If only chemical coupling was involved, phlorizin would inhibit similarly the transport of both phosphate and sodium. Electrical coupling is also suggested by studies conducted in rat proximal tubules *in vivo* (Samarzija *et al.*, 1983).

The sigmoidal dependency of phosphate flux on sodium concentration (Hoffmann *et al.*, 1976; Cheng & Sacktor, 1981; Strevey *et al.*, 1984) is indicative of the involvement of more than one Na^+ ion per phosphate ion transported. The Hill coefficient used in this estimation can result in underestimation of the true stoichiometry, that is if the assumption of strong co-operativity between Na^+ ions is not respected (Kessler & Semenza, 1983). As this assumption has not been verified with the phosphate carrier, we should consider the Hill coefficient of 2 as the minimal value for the actual number of Na^+ ions implicated in phosphate transport.

Phosphate transport at the luminal side occurs against an electrochemical potential difference. The energy required for this active transport is derived from the sodium gradient present *in vivo* across the cell membrane and is associated with conformational changes of the carrier (Béliveau & Strevey, 1987). Since the transport of phosphate is accompanied by translocation of a positive charge, the electrical gradient is favourable and phosphate transport is modulated in the same way as the other substrates transported by the luminal membrane, i.e. stimulated by the presence of an inside-negative potential. This additional driving force should favour accumulation of negatively charged phosphate ions even in the presence of a negative inside potential. The coupling of phosphate transport to the electrical potential is thus obviously a major advantage for reabsorptive properties of the proximal tubules.

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