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Sodium-dependent transport of phosphate by rat liver plasma membrane vesicles

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Plasma membrane fractions were prepared from homogenates of rat liver by density gradient centrifugation and then used for the formation of right-side-out vesicles. Uptake of P_i into the vesicles is rapid when an inwardly directed sodium gradient is present and an overshoot of uptake occurs indicative of accumulation against a P_i concentration gradient. Initial P_i uptake rate in the presence of a K^+ gradient is approx half that seen with Na^+, but uptake in the presence of a choline chloride gradient is very slow. An overshoot does not occur with either K^+ or choline gradients. The $K_m(P_i)$ for the Na-dependent component of P_i uptake is approx. 1 mM and V_{max} at 20°C is 0.8 nmol/min per mg protein. The relationship between initial uptake rate and Na^+ concentration is sigmoid, with a Hill coefficient of 2.6. It is concluded that the cotransporter resembles that of kidney and intestine in possessing at least two interacting sites for Na^+ and that in intact cells the Na^+ gradient maintained by the sodium pump ATPase provides the energy for accumulation of P_i against the unfavourable membrane potential.

Introduction

It has been recognised for several years that phosphate transport across the luminal brush-border membrane of mammalian kidney proximal tubule cells and of intestinal cells is carrier-mediated and is dependent on a sodium gradient [1,2]. Studies of other cell types are now revealing that Na^+-P_i cotransport across the plasma membrane appears to be a general property of most cells and that the activity of the transporters can be increased in response to limited availability of P_i [3–6]. In most physiological conditions, the cytosolic P_i concentration is unlikely to exceed the concentration in extracellular fluid. For rat liver, cytosolic [P_i] is believed to be approximately 1 mM, whereas [P_i] in extracellular fluid is about 2 mM [7]. Thus, the coupling of transport to sodium is probably a device for overcoming the unfavourable barrier to P_i entry represented by the inside negative membrane potential rather than a means for accumulating P_i against a concentration gradient.

Cytosolic P_i is a substrate and/or effector of a number of key enzymes, e.g., glycogen phosphorylase and phosphofructokinase 1. In addition, P_i is a component of the phosphorylation potential expressed by the [ATP]/[ADP][P_i] ratio [8]. For tissues such as liver it has been suggested that the cytosolic P_i concentration is carefully buffered [9] and has potential as a regulator of metabolism. This potential has recently been reviewed [10].

The process by which P_i is taken up by liver cells is likely to be an important component of any physiological mechanism that results in the buffering of the cytosolic concentration. We have therefore been studying P_i uptake by rat hepatocytes and have shown that the process appears to be Na^+-dependent and that it is greatly stimulated by insulin [11]. There have been other reports of the sodium requirement [3,12], but our studies [11] showed that in the absence of insulin stimulation, the rate of P_i uptake by cells suspended in choline or potassium medium was approx. 50% and 60%, respectively, of that seen with cells in Na^+ medium. Also, in the presence of ouabain to inhibit the sodium pump ATPase, the rate of P_i uptake was decreased by only 50%. We decided, therefore, to continue investigation of the characteristics of P_i transport across liver plasma membrane by using membrane vesicles that are preferable in some respects to whole cells for studying transport kinetics.

A fraction of the P_i taken up by whole cells is rapidly incorporated into organic phosphates, particularly when metabolism is stimulated by a hormone such as insulin. Such incorporation could affect the observed kinetics of uptake unless the transport step is always rate-limiting. P_i accumulated by membrane vesicles is not organified and therefore the uptake reflects

transport across the lipid bilayer independent of metabolism.

Experimental

Plasma membrane vesicles

These were prepared from 30 g of fresh liver obtained from 3 or 4 adult male Sprague-Dawly rats (150–200 g) fed on normal rat chow up to the time of killing. The method described by Duffey et al. [13] was followed without modification up to the vesiculation stage and briefly, it involves homogenization of the livers in 1 mM HCO_3 buffer (pH 7.4), followed by centrifugation at $1500 \times g$ for 10 min to isolate a nuclear and plasma membrane fraction. The pellet is dispersed in 56% (w/w) sucrose to give a density of 1.22 and portions of this suspension are layered over sucrose solutions of density 1.18 (40% w/w) and 1.16 (37% w/w) and centrifuged at $66000 \times g$ for 60 min at 4°C. The plasma membranes concentrate at the 1.18–1.16 density interface and are carefully removed with a Pasteur pipette. The fraction is dispersed in 1 mM HCO_3 and washed by centrifugation at $2900 \times g$ for 10 min. The final pellet was taken up in the bicarbonate buffer for enzyme assays or in 300 mM sucrose, 10 mM Hepes-KOH (pH 7.5), containing 10 mM $MgSO_4$ (but, contrary to the published method [13], no $CaCl_2$) for vesicle formation. Vesiculation was brought about by 30 strokes in a tight-fitting Teflon-glass homogeniser followed by one passage through an 18 gauge needle then two passages through a 27 gauge needle. The vesicles formed in the sucrose-Hepes system were stored for up to 1 month in liquid N_2.

Electron microscopy

The vesicles were fixed overnight in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and then collected by centrifugation at $27000 \times g$ for 15 min. They were then treated with 1% osmium tetroxide and stained with uranyl acetate [14]. After dehydration and embedding, ultrathin sections were cut with a microtome.

Enzyme assays

Na^+/K^+-ATPase was measured by a method involving colorimetric determination of liberated P_i [15] and alkaline phosphatase was determined in a 2 ml reaction mixture, containing approx. 0.1 mg or 0.03 mg of protein for the homogenate or vesicles, respectively, at pH 9 and 30°C. The substrate was 2.5 mM *p*-nitrophenyl phosphate in 0.1 M diethanolamine buffer containing 1 mM $MgSO_4$. After 15 min the reaction was stopped with 1 ml of 0.5 M NaOH and the liberated *p*-nitrophenol was determined from the absorbance at 400 nm assuming an extinction coefficient of $18.6 \cdot 10^3\ M^{-1}\ cm^{-1}$.

Protein assay

Protein was assayed [16] using bovine serum albumin as standard.

P_i transport studies

All solutions, including radioactive phosphate were filtered through a Millipore 0.22 μm membrane (catalogue number GSWP 01300) before use in the transport experiments. After thawing, vesicle preparations were passed through a 27 gauge needle and then 0.1 ml of the suspension was mixed with 0.1 ml of 200 mM NaCl made up in 10 mM Hepes-Tris buffer (pH 7.5) containing 50 mM sodium gluconate (buffer A). After equilibration at room temperature (20°C), P_i uptake was initiated by adding 0.05 ml of a $[^{32}P]P_i$ solution to give the required concentrations of 0.4–4 mM (see Results). The level of labelling was approx 2 μCi per 0.05 ml of the P_i solution, i.e., the specific radioactivity ranged from 1 to 10 $\mu Ci/\mu mol$. Uptake was terminated at the appropriate time by the addition of 1 ml of ice-cold stop solution which consisted of 2 mM Na_3AsO_4 in buffer A. After rapid mixing by vortexing, triplicate samples were filtered through 0.45 μm Millipore filters (catalogue number HAWP 02400) under suction. Each filter was washed swiftly by 3×2 ml portions of stop solution and then allowed to dry under the applied suction. The filters were then transferred to Scintran scintillation cocktail (BDH, Poole, UK) for determination of ^{32}P collected on the filter.

Zero-time controls in which stop solution was added to the reaction mixture before the addition of $[^{32}P]P_i$, were performed to correct for radioactivity bound non-specifically to the membrane and for radioactivity trapped on the exterior of the filtered vesicles. Non-specific binding was minimised by soaking the membranes overnight in 10 mM P_i (pH 7.5) before use.

For some experiments, the NaCl and Na gluconate in buffer A were replaced by choline chloride or by KCl plus potassium gluconate. To examine the effect of osmolarity on the amount of P_i accumulated by the vesicles at equilibrium, sucrose was added to buffer A to bring the osmolarity of the incubation mixture to values up to 1 M.

Materials

ATP and *p*-nitrophenol phosphate (disodium salt) for the enzyme assays together with Hepes and Tris buffer materials were obtained from Sigma, Poole, UK. Carrier-free $[^{32}P]P_i$ was obtained from Amersham International, Amersham, UK. General chemicals of Analar grade were supplied by BDH, Poole.

Results

Electron micrographs showed the vesicle preparation to consist of closed vesicles ranging from 0.4 to 0.9

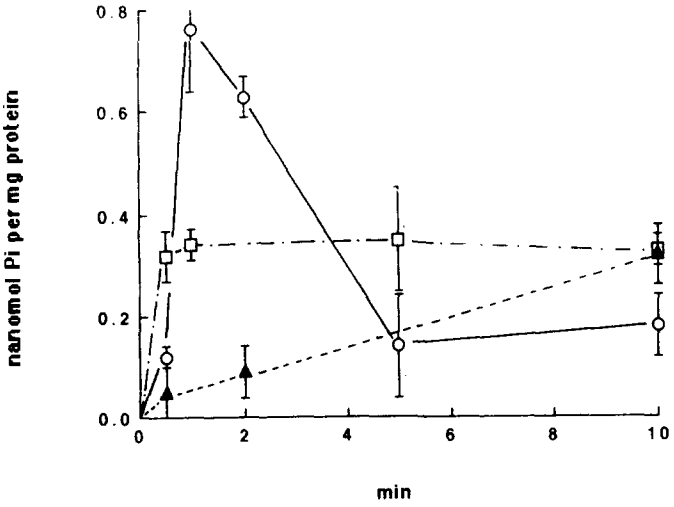

Fig. 1. Uptake of P_i by rat liver plasma membrane vesicles. The vesicles were suspended at 20°C in medium containing 0.6 mM P_i and 100 mM NaCl, ○———○; 100 mM KCl, □-·-□; or 100 mM choline chloride, ▲. The points are the means (plus the range shown by error bars) of results obtained in two separate experiments performed in triplicate. All data have been corrected for non-specific binding.

μm in diameter. There was virtually no contamination by mitochondria and little non-vesicular material was present. Na^+/K^+-ATPase and alkaline phosphatase had specific activities of 19 ± 2.0 (six preparations) μmol/h per mg and 10 ± 1.8 (three preparations) μmol/h per mg, respectively. The ATPase value is very close to that reported previously for this method [13], but our alkaline phosphatase value is about 3-times greater than that in the earlier report. The specific activities in our preparation were enriched 3- to 7-fold relative to the homogenate, but this is considerably less than the 15- to 18-fold reported by Duffey et al. [13]. There is no obvious explanation of this discrepancy given that the specific activities in our final preparation were so similar to the published values.

Uptake of P_i by the vesicles (corrected for non-specific binding that typically amounted to 0.05 nanomol) from 0.6 mM P_i is shown in Fig 1. In the presence of an NaCl gradient (inside < outside) there is a rapid uptake of P_i that peaks at 1 to 2 min followed by an efflux, i.e., the vesicles demonstrate an overshoot [17] characteristic of accumulation against a concentration gradient. When the Na^+ gradient was replaced by K^+, the vesicles took up P_i at an initial rate that was rapid, but at 1 min the take up was only half that seen with Na^+ and there was no clear evidence of an overshoot. Uptake in the presence of a choline chloride gradient was relatively slow and almost linear with time to reach the same level as that seen with K^+ after 10 min, and similar to the Na^+ value at this time point. Entry of P_i into the vesicles in the presence of choline is assumed to derive from passive transporters and and/or non-carrier-mediated leakage.

The effect on the uptake at 2 min of varying the P_i concentration in the bathing medium is shown in Fig. 2a. In the presence of an inwardly directed Na^+ gradient, the uptake shows saturation kinetics but when the Na^+ was replaced by choline, the uptake rate was first order with respect to P_i concentration. A replot of the Na^+-dependent fraction of uptake in the form of a Hanes plot of $[P_i]/v$ against $[P_i]$ is shown in Fig 2b. The data are moderately well fitted by the linear plot and calculation of the kinetic parameters by weighted regression analysis [18] gave values of 0.63 ± 0.44 mM and 0.78 ± 0.30 nmol/min per mg for K_m and V_{max} respectively.

The effect on P_i uptake of changing the magnitude of the Na^+ gradient is shown in Fig 3. The plot is sigmoid, which is indicative of positive cooperativity

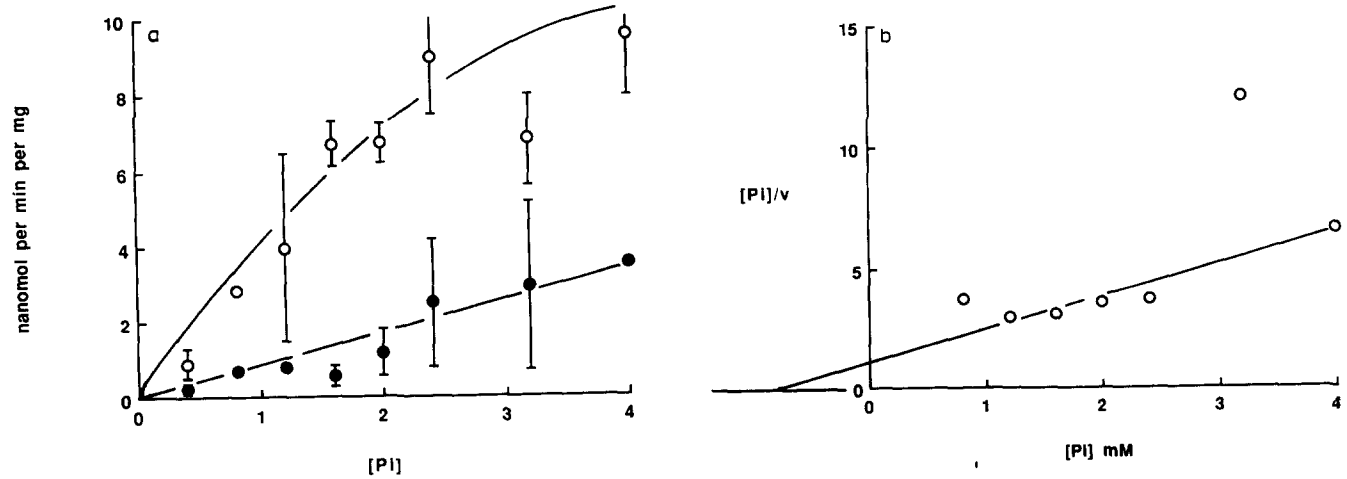

Fig. 2. Concentration dependence of P_i uptake. (a) Vesicles were suspended in medium containing P_i of the indicated concentrations plus 100 mM NaCl (○) or 100 mM choline chloride (●). Uptake was allowed to proceed for 2 min before addition of the stop solution. The data points and SEMs were calculated from four separate experiments performed in triplicate except for the choline value at 4 mM P_i, which is the mean of values obtained in two experiments. (b) Hanes $[P_i]/v$ against $[P_i]$ plot of the Na^+-dependent fraction of the uptake shown in (a).

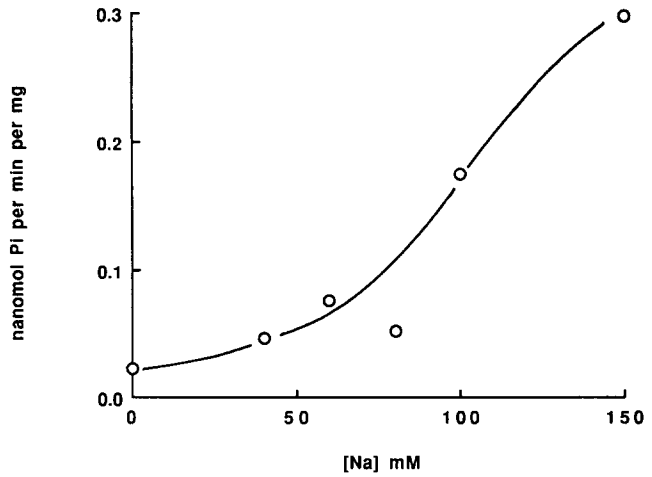

Fig. 3. Effect of sodium concentration on P_i uptake by plasma membrane vesicles. The P_i concentration was 0.6 mM throughout and choline chloride was added to the incubation mixtures to adjust the total NaCl + choline chloride concentration to 150 mM in each case. The data points are mean values determined in two separate experiments.

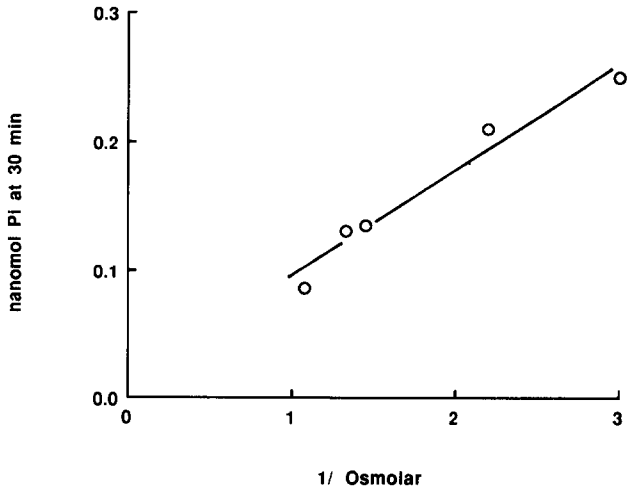

Fig. 5. Effect of medium osmolarity on the amount of P_i taken up per mg of vesicle protein after incubation for 30 min with 0.6 mM P_i. The data points are means of two experiments performed in triplicate.

between multiple Na^+ binding sites. Replotting of the data (corrected for the 'passive' and non-carrier-mediated components observed at zero $[Na^+]$) as a Hill plot generated a linear graph with a slope of 2.6 and $[Na]_{0.5}$ of approx. 110 mM (Fig. 4).

Vesicles containing 300 mM sucrose that were suspended in uptake media containing sucrose in sufficient concentrations to bring the osmolarity up to 1 osM and then incubated for 30 min with P_i before measurement of the accumulated P_i, were used to distinguish between binding to the membrane and uptake into the vesicles. Total uptake at 30 min (assumed equilibrium) was proportional to the reciprocal of the osmolarity of the bathing medium, indicating uptake of P_i into an osmotically active space (Fig. 5).

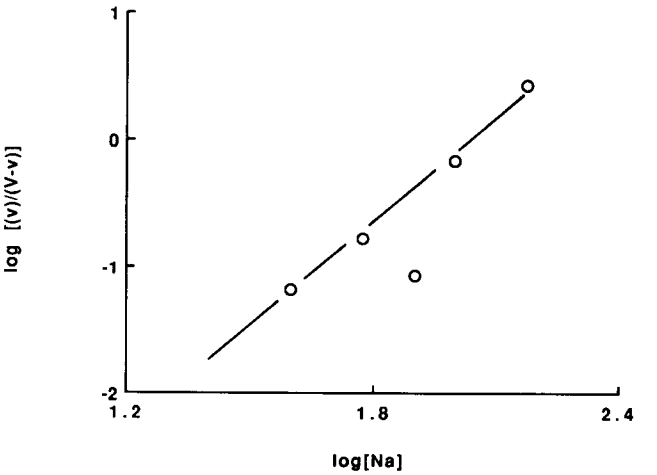

Fig. 4. Hill plot of $[Na^+]$-dependency. For calculating log $[v/(V_{max} - v)]$ values plotted on the y axis, V_{max} at 0.6 mM P_i was estimated from the data in Fig. 3 by extrapolation and assumed to be 0.4 nmol/min per mg. (Calculated slope by regression is 2.6 ± 0.47 with $r = 0.89$).

Discussion

The results reported here demonstrate that the plasma membrane of hepatocytes contains a phosphate transport system that is sodium-dependent. A functioning sodium pump ATPase in intact liver cells is therefore able to drive the uptake of the P_i anion in spite of the inside-negative membrane potential. Demonstration of an overshoot shows that the system is capable of transporting P_i against a concentration gradient, even though such transport may not be called for in vivo because the cytosolic concentration is unlikely to exceed the normal plasma P_i level [7,10]. A K^+ gradient stimulates P_i uptake by the vesicles but is unable to produce an overshoot. It is not possible to decide whether all of the measurable uptake in the presence of choline derives from leaky membrane vesicles or whether significant uptake of P_i occurs in these vesicles, and therefore by implication in liver cells, by non-Na^+-dependent transporter(s). In studies with whole cells it is found that only the Na^+-P_i fraction of uptake is stimulated by insulin [11], suggesting that the cotransport system is the one that is most important for meeting physiological demands for P_i.

The K_m value for Na-P_i cotransport is approx. 0.6 mM, which is essentially identical to the value of 0.7 to 0.9 mM obtained with whole cells [11] but considerably greater than the K_m of approx. 0.1 mM reported for membrane vesicles prepared from the brush border of

renal proximal tubules and from the small intestine [2]. The V_{max} of 0.35 nmol/min per mg protein found for liver vesicles is comparable with that for intestinal preparations but only about 1/7th of the activity of proximal tubule vesicles [2].

In common with other Na-P_i cotransporters that have been characterised, the liver protein exhibits co-operativity with respect to sodium. The Hill coefficient for the liver transporter calculated from the vesicle data is 2.6, suggesting a minimum of three interacting sites for Na^+ ions. The kidney and intestinal transporters are assumed to transport 2 Na^+ ions per phosphate anion [1,2] and data obtained for whole hepatocytes suggests a similar stoichiometry [11,12]. For whole cells, $[Na]_{0.5}$ is approx 80 mM compared with 110 mM for vesicle transport. The values for the Hill coefficient and $[Na]_{0.5}$ depend on the estimate of V_{max} (for substitution into the Hill equation). This is difficult to perform precisely from a sigmoid curve and it is not surprising therefore that the vesicle and whole cell data do not match exactly. Despite this uncertainty however, our data emphasise the great importance of an Na^+ gradient for P_i transport into liver cells.

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