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Uptake of phosphate by rat hepatocytes in primary culture: a sodium-dependent system that is stimulated by insulin

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Primary cultures of rat hepatocytes take up phosphate by a saturable Na^+-dependent process. Thus the plasma membrane possesses an Na^+-P_i cotransporter of the type described for many cell types, e.g., kidney proximal tubular cells and enterocytes. Coupling to Na^+ overcomes the barrier to anion entry represented by the membrane potential. At 0.12 mM P_i, the effect of Na^+ is cooperative with a Hill coefficient of 1.7 suggesting two sodium sites per molecule of carrier. At 37°C, the K_m (for P_i) and V_{max} for the sodium-dependent fraction of P_i uptake are approx. 1 mM and 0.35 nmol P_i/min per mg cell protein, respectively. Insulin stimulates V_{max} four-fold with no significant effect on K_m. P_i uptake in the absence of sodium is not affected by insulin. The stimulation by insulin could be of metabolic significance. Glucose phosphorylation at the expense of ATP is raised in liver following insulin stimulation, and thus, initially there may be an increased demand for P_i for oxidative phosphorylation until new steady-state conditions of hexose phosphate concentrations and of ATP turnover become established.

Introduction

The plasma phosphate concentration in mammals is normally maintained within narrow limits. The major point of control seems to operate at the kidney where alterations in the proximal tubular reabsorption rate occur in response to hormones, particularly parathyoid hormone, and to dietary availability of phosphate [1,2].

All cells require a continuous supply of P_i from plasma to meet the needs of cell metabolism. Cytosolic P_i is a component of the phosphorylation potential which may be calculated from the (ATP)/(ADP)(P_i) ratio [3,4] and P_i is also a substrate and/or effector for a number of key cytosolic enzymes, e.g., glycogen phosphorylase and phosphofructokinase 1. Whether P_i has a direct regulatory role in metabolism is controversial, but considerable evidence has now accrued to indicate that cytosolic levels of P_i are normally closely buffered (see Ref. 5 for an up-to-date review of this subject). It seems probable therefore, that cellular control mechanisms will exist to maintain the cytosolic concentration within narrow limits. Such controls would be secondary to the 'gross' regulatory mechanisms operating to keep plasma P_i concentrations within the normal physiological range. The central metabolic role of the liver suggests that P_i availability is likely to be particularly important for this tissue.

Release of P_i from the relatively large amount of P_i stored within mitochondria [4,6] is probably of some importance in buffering cytosolic P_i but transport of P_i across the plasma membrane of liver cells is likely also to be significant in stabilising cytosolic levels [7]. It was shown 70 years ago that injection of insulin into rabbits caused a rapid fall in the blood P_i concentration [8] and it was suggested that the hormone increases the uptake of P_i by the tissues. This conclusion was borne out by a number of subsequent studies that indicate that insulin increases the accumulation of P_i in various cell types notably heart muscle [9,10] and adipose tissue [11]. Medina and Illingworth [9,10] concluded that P_i accumulates in heart muscle because insulin decreases the efflux of P_i. A similar conclusion was reached for liver [12] but direct measurements of efflux rates from hepatocytes [13] do not support the theory that insulin affects efflux.

The importance of insulin in regulating liver metabolism suggests that the hormone may also influence P_i utilisation in this tissue. The increase in glucokinase activity that results from insulin action could initially raise the demand for ATP, and thus, require an increase in the availability of P_i for oxidative phosphorylation until new steady-state conditions for the concentrations of metabolites such as hexose phos-

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phates and for ATP become established. Plasma membrane transport systems for P_i have been studied for several tissues, particularly intestine and kidney [1,2] but liver has received relatively little attention. Uptake of P_i by a cell line derived from hepatoma has been shown to be Na^+-dependent and to involve a transporter that is sensitive to thiol-reactive reagents [15].

Using isolated hepatocytes in primary culture we are studying liver membrane transport systems for P_i and the means by which it/they may be regulated. Our aim is to gain understanding of the part that transport plays in maintaining cytosolic P_i levels and in meeting the metabolic demand for P_i in liver.

Materials and Methods

Isolation and culture of hepatocytes. The cells were prepared by collagenase-hyaluronidase digestion of liver slices under sterile conditions in Hanks' medium essentially as described by Fry [16] but with the following modifications: During the digestion stage, extra buffering of the enzyme solution was achieved by the inclusion of 36.6 mM Hepes, as well as the components listed in the original method [16]. The solution was brought to pH 7.6 by the addition of NaOH and the concentration of $MgCl_2$ was 1.54 mM. At all stages of the preparation, pure oxygen was blown over the surface of the cell suspension contained in Corning disposable 25-cm^2 polystyrene cell culture flasks which were shaken continuously and maintained at 37°C.

At the harvesting stage, the cells were pelleted by centrifugation in an MSE Centaur centrifuge that was accelerated rapidly to 2000 rpm, maintained at this speed for 30 s and then allowed to stop. Approx. $6 \cdot 10^7$ cells were obtained from 8 to 10 g liver and were cultured in four Corning disposable flasks under a humidified atmosphere containing 5% CO_2 [15]. The method differed from the published procedure in that dexamethazone was not used and insulin was present at 10^{-7} M. After 2 to 3 h the medium was replaced by insulin-free medium and the cells were kept in culture for at least one day before use in transport experiments. Hepatocytes do not become firmly attached to plastic and can be removed by agitation. Treatment with trypsin is not required. The cells remained suitable for transport experiments for up to 4 days following isolation. The viability of resuspended cells was tested with trypan blue and the fraction excluding the dye was greater than 95%. This was true for cells tested immediately after separation and for cells kept in culture conditions for up to 4 days. Hepatocytes do not proliferate in primary culture but appear to retain their biochemical features for about one week [15].

Measurement of uptake. A system developed for studying P_i uptake by proximal tubule cells [17] was applied to hepatocytes with further improvements. Uptake was initiated by the addition of carrier-free [^{32}P]P_i (approx. 5 μCi/3 ml to cells suspended in Krebs-Ringer-Bicarbonate (KRB) medium containing P_i, Na^+, choline, etc., at the required concentrations (see Results)). KRB is prepared fresh daily by mixing 0.154 M stock solutions of the following components in the proportions indicated in brackets: NaCl (100), KCl (4), KH_2PO_4 (1), $MgSO_4$ (1), $NaHCO_3$ (21). Three volumes of 0.11 M $CaCl_2$ are then added plus a few drops of a phenol red solution and the whole solution gassed with 95% O_2/5% CO_2 for at least 20 min at 37°C to bring the pH to 7.4. Glucose was added to a final concentration of 10 mM. After gassing, bovine serum albumin was added at a level of 1 mg/ml and the cell density was 2 to 4 mg cell protein/ml (2 mg/ml correspond to $(1.5–2) \cdot 10^6$ cells per ml). Triplicate 0.2-ml samples were removed at 45-s intervals up to 3 min and the cells separated from the bathing medium by microcentrifugation for 1 min at $8700 \times g$ through silicone oil/dinonyl phthalate (3:2) into $HClO_4$. The microcentrifuge tubes were fitted with inserts fashioned from large disposable pipette tips to prevent the radioisotope from coming into contact with the walls of the centrifuge tube to which P_i will adhere. The medium and the bulk of the oil were removed by aspiration and the inserts were discarded. The rest of the tube containing the cell pellet in $HClO_4$ was transferred to a vial containing Scintran cocktail for determination of the radioactivity by scintillation counting. In the original method [17], the tube needed to be sliced at the level of the oil so that the upper part that had been in contact with radioactive medium could be discarded. The use of a disposable insert makes slicing unnecessary and in our experience, greatly improves the precision of the transport assay.

The initial rate of uptake is calculated from the slope of a computer-generated linear plot of uptake against time using simple least squares regression.

For those experiments in which the concentration of P_i and/or the univalent cations differed from the concentrations normally present in KRB, a portion of the cells was washed twice in modified KRB immediately before the uptake experiment and the cells were then transferred to incubation media containing the required concentration of P_i and Na^+ ions. The modified KRB lacked P_i or was prepared by replacing Na^+ ions by choline as appropriate for the particular experiment. The cells were exposed to the modified KRB for 3 to 5 min in total.

In experiments to test the effect of insulin on uptake, the hormone (10^{-7} M) was included in all the media used for washing of the cells after harvesting and was also present at the same concentration during uptake measurements. Following the washing procedure (approx. 5 min duration) the cells were incubated for a further 5 min in the uptake medium containing

insulin before initiating the uptake with the radioisotope.

At early stages of the investigation, ^{3}H-labelled inulin was included in some incubation mixtures to allow calculation of the volume of medium trapped by the cells during microcentrifugation. The amount of non-internalised P_i adhering to the cells which is made up of the fraction bound to the cell membrane plus that present in the trapped medium, was estimated by addition of cells to medium containing 10 mM arsenate (stop solution) followed by immediate centrifugation through oil. The arsenate was present to inhibit any P_i transport by a specific carrier.

Protein estimation. The biuret method [18] was used with bovine serum albumin as standard.

Materials. Collagenase and hyaluronidase were obtained from Boehringer-Mannheim (Lewes, UK). Silicone oil AR200 was obtained from Wacker Chemie (Walton-on-Thames, UK) and other chemicals from Sigma or BDH (Poole, UK). Cell culture materials were obtained from ICN Flow (High Wycombe, UK) and from Gibco BRL (Uxbridge, UK).

Results

Fig. 1 shows a typical uptake plot and confirms that P_i uptake by the cells is linear for up to 3 min, both in the presence and absence of insulin. The figure shows that under conditions where near maximal rates of uptake occur (in this case at 1.5 mM P_i in the presence of insulin) linear rates of uptake are still observed over a 3 min period. Uptake remains linear for up to 10 min

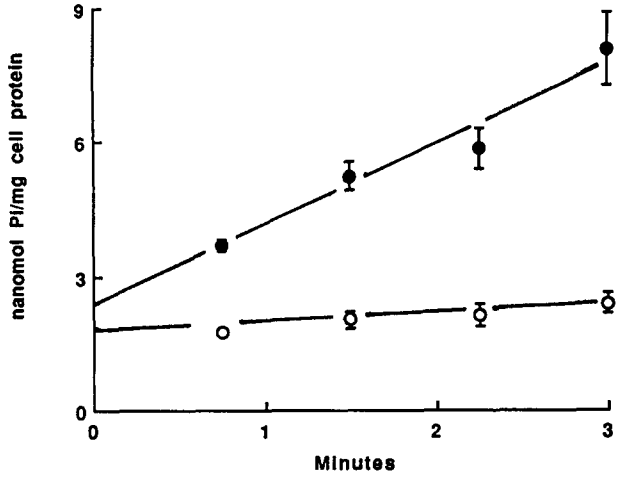

Fig. 1. Uptake of P_i by hepatocytes. The cells were incubated at 37°C in medium containing P_i at 1.19 mM (○), or 1.5 mM P_i plus 10^{-7} M insulin, (●). The points are the mean plus S.E. values of six determinations of the P_i accumulated by the cells. The rate of uptake is obtained from the linear regression coefficient ($r = 0.983$ for (○) and 0.978 for (●)). The intercept value indicates that 1.3 and 1.5 μl of medium is trapped per mg of cell protein in the absence and presence of insulin, respectively.

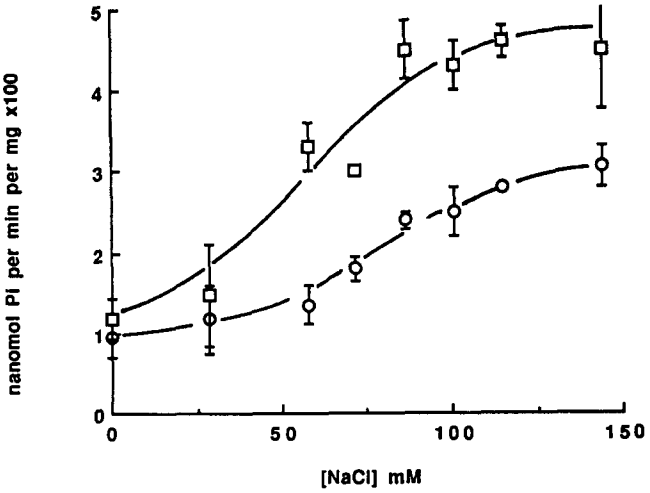

Fig. 2. Effect of Na^+ ions on the rate of P_i uptake. The rate of uptake was measured at various [Na^+]. Choline chloride was added in sufficient quantities to ensure that the total Na^+ plus choline concentration was 143 mM in each case. (○), Uptake at 0.12 mM P_i; (□), uptake at 0.12 mM P_i by insulin treated cells. Mean and S.E. values calculated from three separate experiments with different cell batches.

with most cell preparations, but in some instances uptake plots curved upwards beyond 4 min. This was particularly so for cells that had been cultured. The cause of the non-linearity was not investigated further but similar behaviour has previously been noted for P_i uptake measurements with cultured hepatocytes [15]. At least up to day 4, the number of days in culture had no detectable effect on transport behaviour of the cells, but no experiments were conducted with cells older than this.

Experiments with labelled inulin indicated that at the cell concentration used, approx. 1–2 μl of medium was trapped in the cell pellet deposited in the $HClO_4$ layer. When pelleting cells from normal KRB (containing 1.19 mM P_i) the membrane bound (i.e., non-internalised) P_i was negligible compared with the labelling present in the trapped medium and a reliable estimate for membrane-bound P_i could not be obtained. The use of stop solution to estimate the non-internalised plus trapped P_i showed that the value predicted for the quantity of labelled P_i in the cells at zero time (i.e., the intercept value in Fig. 1) agreed very closely with the value obtained by direct measurement. In the experiment shown in Fig. 1 for example, the intercepts indicate that 1.3–1.5 μl of medium was trapped per mg cell protein; a result that is in good agreement with the earlier measurements using inulin. Therefore, as uptake was always determined from a time-course experiment, the practice of direct determination of the zero-time labelling was discontinued.

Fig. 2 shows the effect of Na^+ ion concentration on the initial rate of uptake of P_i determined at 0.12 mM P_i. The figure also shows some data obtained with cells

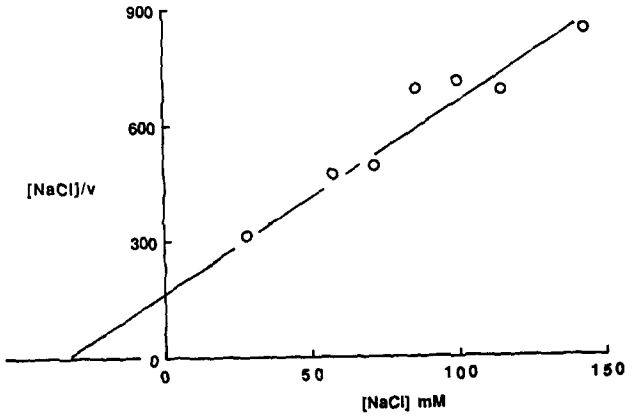

Fig. 3. $[Na^+]/v$ against $[Na^+]$ (Hanes) plot of of uptake at 0.6 mM P_i. The sodium-dependent component of uptake at each $[Na^+]$, v, was calculated by subtracting the value of uptake rate at zero $[Na^+]$.

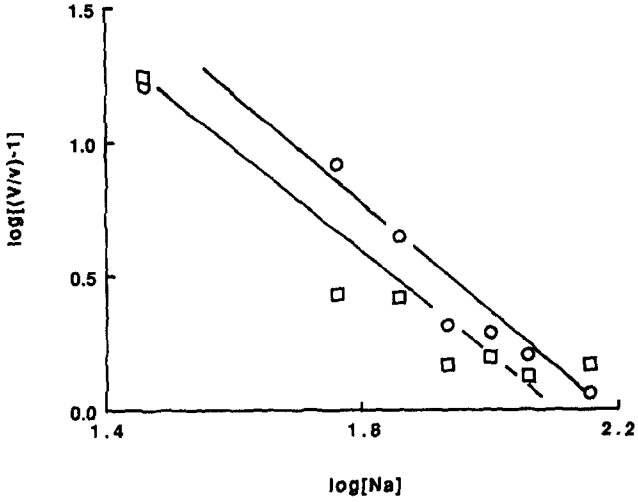

Fig. 4. Hill plot of the uptake data at 0.12 mM P_i. $\log[(V_{max}/v)-1]$ is plotted against $\log[Na^+]$. (○), Untreated cells; (□), insulin-treated. The Hill coefficients (maximum slope) were calculated by linear regression ignoring the highest and lowest data points for each line.

that had been incubated for 10 min at 37°C in normal KRB containing 10^{-7} M insulin. Subsequent washing of the cells and the uptake measurements were performed in media that also contained the hormone at the stated concentration. At 0.12 mM P_i the response of uptake to Na^+ concentration is sigmoid. Insulin treatment did not affect the sigmoid shape of the plot but resulted in stimulation of the uptake at all Na^+ concentrations. $[Na]_{0.5}$ is 65–80 mM. At 0.6 mM P_i, cooperativity is much less obvious and the relationship between uptake rate and $[Na^+]$ approximates to Michaelis-Menten kinetics. A plot of the Na^+-dependent component of uptake in the form of $[S]/v$ against [S] (Hanes plot) for 0.6 mM P_i is shown in Fig. 3. The plot is linear and calculation of the kinetic parameters by weighted regression analysis [19] gives an apparent K_m for sodium of 41.7 ± 15 mM with an apparent V_{max} of 0.21 ± 0.026 nanomol P_i/min per mg cell protein. 'Apparent' constants are obtained because the P_i concentration is non-saturating.

The sodium-dependent component of uptake at 0.12 mM P_i is shown as a Hill plot in Fig. 4. For both basal cells and insulin treated, the data fit well to the plot

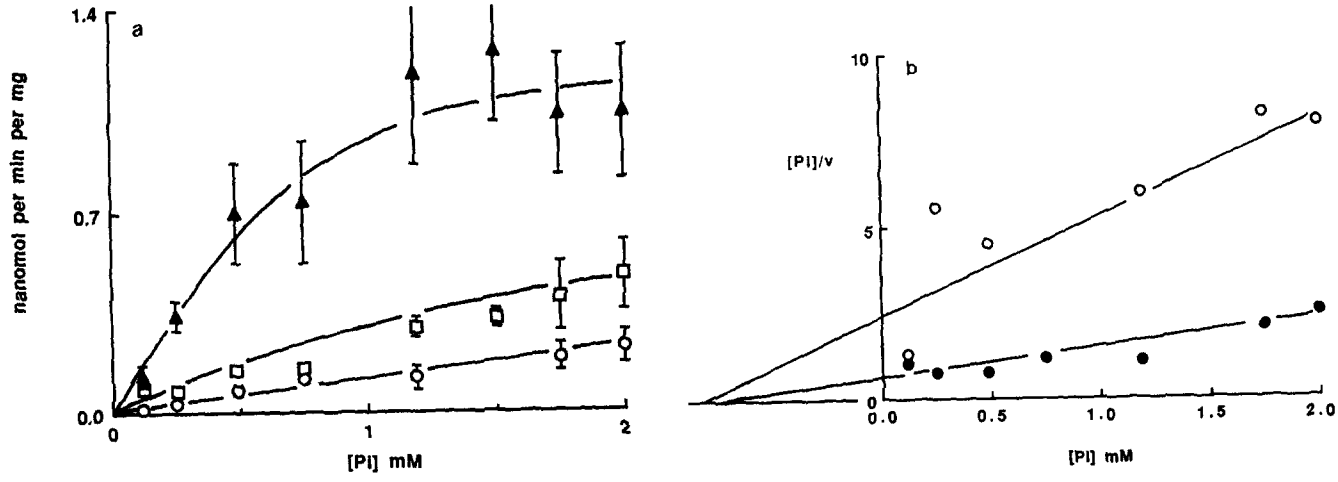

Fig. 5. Concentration dependence of P_i uptake. The hepatocytes were washed in P_i-free medium or choline medium as appropriate immediately before uptake measurements. For insulin-treated cells, the P_i-free or choline wash solution contained 10^{-7} M insulin and the cells were incubated for a further 5 min at 37°C in medium of the required P_i plus insulin before the addition of radiolabel to begin the uptake measurement. (a) (○), Uptake in presence of choline, (□), uptake by untreated cells in the presence of Na^+; (▲), uptake by insulin-treated cells in the presence of Na^+. The data points are the mean ± S.E. of triplicate estimations performed on four different batches of cells. The choline data are from four experiments with untreated cells plus three experiments with insulin-treated cells. As there was no detectable difference between them, the data were grouped and the errors calculated from the results of seven batches of cells. (b) Hanes $[P_i]/v$ against $[P_i]$ plot of the Na^+-dependent fraction of uptake shown in (a). The lines are drawn according to the kinetic constants quoted in the text that were calculated by a weighted regression fit to the Michaelis-Menten equation. (○), Untreated cells; (●), insulin-treated cells.

(correlation coefficients are 0.94 and 0.93 for treated and non-treated, respectively) with mid point slopes of 1.62 ± 0.22 (insulin-treated) and 1.7 ± 0.14 (non-treated) that did not differ significantly. Thus, there are likely to be two interacting sites for sodium per molecule of carrier.

The effect of varying the concentration of P_i in the uptake medium on the initial rate of uptake by the cells is shown in Fig. 5. Uptake in the presence of choline was linear with P_i concentration and no difference could be detected in the rate of uptake between untreated cells and those treated with insulin. Uptake in the presence of Na^+ ions is saturable and is markedly stimulated by insulin. The calculated K_m values [19] for the Na^+-dependent component of uptake are 0.94 ± 0.18 mM and 0.7 ± 0.33 mM for untreated and insulin-treated, respectively. The equivalent V_{max} values are 0.354 ± 0.04 nanomol/min per mg and 1.37 ± 0.26 nanomol/min per mg. Thus, insulin stimulates the rate of P_i transport four-fold with no significant effect on K_m. The data are presented as Hanes plots in Fig. 5b.

Li^+ ions were found to be approximately 60% as effective as Na^+ in supporting uptake at 2 mM P_i. Incubation of the cells for 10 min at 37°C with 1 mM ouabain prior to measuring the P_i uptake at 1.19 mM P_i inhibited uptake by $39\% \pm 20\%$ (3 determinations in duplicate with different batches of cells) relative to untreated controls. The lithium and ouabain results were obtained in the absence of insulin.

Discussion

At the start of our investigation we used freshly prepared hepatocytes for studying P_i transport and the sensitivity of the latter to hormones. The transport properties of each batch of cells varied greatly however, and refining the transport parameters proved to be very difficult. We therefore decided to culture the cells for 1 to 4 days before using them for transport. Such treatment is reported to restore cellular ATP levels in hepatocytes [15] but can be disadvantageous in studies of hormonal effects on transport because the established culture procedures recommend the inclusion of insulin and dexamethazone in the culture medium. This difficulty was overcome by including insulin in the medium for only the first few hours of culture by which time the cells have adhered to the culture flask.

P_i uptake is stimulated by Na^+ ions and the Na^+-dependence at low P_i concentrations exhibits sigmoid kinetics. A Hill plot of the data suggests that two Na^+ ions are tranported per phosphate ion. If the prime reason for Na^+-P_i cotransport is to overcome the unfavourable condition for P_i entry represented by the membrane potential, $H_2PO_4^-$ is probably the preferred species for transport but HPO_4^{2-} could be transported electroneutrally. $[Na]_{0.5}$ is approx. 70 mM. A previous report [15] gives a value of 34 mM which is very similar to the result we obtained for the apparent K_m at a P_i concentration of 0.6 mM where the cooperativity is less noticeable.

The dependence of uptake of P_i on concentration is saturable in the presence of Na^+ ions. There is significant uptake of P_i by the cells when sodium is replaced by choline but the rate is first order with respect to $[P_i]$. K_m for the Na^+-dependent fraction is approx. 1 mM and V_{max} is 0.35 nanomol/min per mg cell protein. At a concentration of 2 mM, a value close to the plasma concentration for rats, uptake in the presence of choline is 50% that of the Na^+-supported rate. This result is nearly identical with the figure given for a hepatoma cell line [14] and for a rat hepatocyte primary culture [15].

These results may cast doubt on the significance of sodium in P_i transport in liver until the effect of insulin is taken into account. Insulin stimulates Na^+-dependent uptake four-fold with no significant effect on K_m. Choline-supported uptake is not affected by insulin. Anabolic actions of the hormone are expected to increase the incorporation into organic metabolites of any P_i entering the cells. If P_i entry is critically dependent on the concentration gradient for the anion, any lowering of the cytosolic concentration by metabolic trapping would result in an increased accumulation of labelled P_i. The cytosolic P_i concentration is believed to be approx. 1 mM [4,5] and thus at an extracellular concentration of 2 mM, a favourable gradient appears to exist for P_i entry. This conclusion ignores the effect of the membrane potential however, which is estimated to range from -24 mV to -47 mV [20]. If P_i distributes across the membrane passively, at a membrane potential of -40 mV, the In/Out concentration ratio across the membrane at equilibrium would be 1:4.8 for singly-charged phosphate and 1:22.7 for HPO_4^{2-}. The actual value for cytosolic P_i is therefore considerably in excess of the equilibrium value, and hence, it is unlikely that a fall in cytosolic P_i consequent to metabolic trapping can provide sufficient driving force to account for the large increase in P_i uptake in the presence of insulin. Similarly, dilution of cytosolic P_i accompanying insulin-induced swelling [21] can hardly contribute to the effect of insulin on P_i uptake.

Preliminary experiments established that exposure of the cells to 10^{-7} M insulin for 10 min elicited a maximum and reproduceable response. This hormone concentration seems high relative to physiological conditions but the level of hormone reaching the liver via the portal blood system is likely to be considerably higher than that which is detected in the general circulation. In addition the liver is a major site of insulin degradation and hepatocytes in culture have been shown to bring about significant breakdown of insulin

within 15 min of incubation [22]. Insulin turnover during our transport experiments could account to some extent for the relatively high hormone concentration needed for maximum effect. The possibility cannot be ruled out, however, that at least part of the observed insulin effect could have been mediated via stimulation of the IGF-1 receptor. Study of the effects of various growth factors on liver P_i transport needs to be undertaken.

The greatly enhanced uptake presumably only becomes possible by linking with sodium to overcome the unfavourable condition for anion entry represented by the membrane potential (negative inside). If the cultured cells are a true reflection of the liver in vivo, it is conceivable that the P_i supply to liver cells is almost entirely dependent on insulin, presumably by an increase in the number of transport sites or by stimulation of existing molecules of carrier. Insulin stimulates Na^+-P_i cotransport in renal proximal tubule and it has been suggested that the renal adaptation to dietary deprivation of P_i is insulin dependent [23]. Stimulation by insulin of the Na^+K^+-ATPase could contribute to the increased P_i uptake by raising the concentration gradient for Na^+ ions. Such action would be expected to lower the $[Na]_{0.5}$ value. Fig 4 shows that any decrease is small and is probably not very significant. The activity of Na^+ in the cytosol is 12.2 mM [20], i.e., less than one tenth of extracellular, and so it is questionable whether further lowering of the intracellular concentration could greatly accelerate the Na^+-P_i cotransport system.

The liver continues to need P_i for ATP synthesis and metabolism under gluconeogenic conditions that are associated with a lowered insulin/glucagon ratio. Should an increased demand for P_i arise in these circumstances, agents other than insulin must be involved in the stimulation of uptake. Glucagon has been shown to mobilise P_i from tissues into blood plasma in mice and it has been suggested that this hormone plays a role in counteracting the hypophosphataemia associated with fasting [24]. Thus, insulin is probably only one of a number of hormonal agents that control P_i transport in liver.

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