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Inhibition by cyclic AMP and phorbol esters of sodium-dependent uptake of phosphate by rat hepatocytes

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Abstract

Na^+-dependent phosphate uptake by rat hepatocytes in primary culture is inhibited in a time-dependent fashion by cyclic AMP and by the myristate, acetate ester of phorbol. After incubation for 15 min at 37°C with 10^{-7} M dibutyryl cAMP, the V_{max} of transport is decreased from 0.52 to 0.23 nmol P_i/min per mg protein but the K_m value of approximately 1 mM is hardly affected by the treament. Thus, physiological control of P_i uptake by liver cells probably involves protein phosphorylation(s) catalysed by protein kinases. Protein kinase C may be important but the relatively high concentration of phorbol ester needed to cause inhibition of transport is not convincing evidence for protein kinase C involvent. In the presence of fructose, the rate of P_i uptake is decreased by 50%. This effect is probably secondary to a depletion of cellular ATP.

Key words: Phosphate transport; Hepatocyte; cyclic AMP; Phorbol ester; Fructose metabolism

1. Introduction

A supply of phosphate to the tissues is essential for ATP production by oxidative and substrate-level phosphorylation. The cytosolic P_i concentration, which in hepatocytes is reported to be approximately 1 mM [1] seems to be kept within fairly narrow limits and therefore it must be assumed that utilisation of P_i by incorporation into metabolites, either directly or indirectly via ATP, plus sequestration into mitochondria [2] is balanced by cellular uptake of the anion together with that released by phosphate ester hydrolysis and from mitochondrial efflux [2,3]. Under circumstances in which metabolism is stimulated, it is likely that the cellular demand for P_i from extracellular fluid will be increased until new steady state conditions of P_i utilisation and release from metabolites becomes established. Phosphate depletion would be expected to interfere with normal liver metabolism

Recently, we demonstrated that P_i transport into rat hepatocytes occurs by a sodium-dependent transport system that is greatly stimulated by insulin [4,5] and proposed that the physiological significance of the insulin action resides in the greater need for P_i consequent to the stimulation of metabolism by the hormone. Phosphate depletion of a cultured cell line derived from hepatoma also leads to a stimulation of P_i transport capacity [6]. The response to low P_i seems to be a property of all Na^+-P_i cotransport systems but is especially well documented for the transporter in the proximal tubule of mammalian nephrons [7,8]. The liver plays a central role in the metabolism of the whole animal and is a target tissue for numerous hormones, e.g., glucagon, catecholamines and glucocorticoids. If the P_i supply is adjusted to meet altered metabolic demands for the anion it is possible that transport into hepatocytes is subject to regulation by a number of factors of which insulin is one example. We report here on results we have obtained in studies of the action of various effectors on P_i transport into hepatocytes.

2. Materials and methods

Primary culture of hepatocytes. Cells were isolated from slices of fresh rat liver by collagenase-hyaluronidase digestion essentially as described by Fry [9] but with the modifications given in our earlier paper [4].

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The washed cells were cultured under an humidified atmosphere containing 5% CO_2 at 37°C [4,10]. Approximately $1.5 \cdot 10^7$ cells were placed in 25-cm^2 polystyrene culture flasks and the medium contained 10^{-7} M insulin. The medium was replaced by insulin-free medium after 3 h and the cells were maintained in these conditions for at least 24 h before use in transport experiments. When cells were kept in culture for up to one week, the medium was changed every 48 h; each time with insulin-free medium. Cell viability was tested routinely by Trypan blue exclusion.

Measurement of P_i uptake. The cells were suspended in Krebs-Ringer-Bicarbonate (KRB) medium containing P_i, Na^+, etc., at the concentrations needed for particular experiments (see Results) plus bovine serum albumin at 1 mg/ml. The cell density was approximately $2 \cdot 10^6$ per ml. Uptake was inititated by the addition of 5 μCi of carrier-free [^{32}P]P_i/3 ml of cell suspension. Triplicate samples (0.2 ml) were withdrawn at 45 s intervals up to 3 min and the cells separated from the bulk of the labelled medium by microcentrifugation through silicone oil/dinonyl phthalate [4]. The rate of uptake is calculated from the slope of the plot of accumulated radioactivity against time.

For experiments in which the concentration of P_i was varied, cells suspended in normal KRB containing P_i at 1.19 mM were washed twice in modified KRB immediately before transport and then transferred to vials containing medium of the required concentration. A similar washing procedure was used when Na^+ ions were replaced by Li^+.

Treatment with cyclic AMP and phorbol esters. The cells were pre-incubated for timed periods with the concentrations of the agents at the concentrations indicated in Results before the initiation of P_i uptake. The agents were present during the uptake period at the same concentrations used in the pre-incubations. The results were compared with those obtained with control incubations that lacked cyclic AMP or phorbol.

Treatment with fructose. The cells were incubated for approximately 40 min in KRB medium containing 10 mM fructose in place of glucose. P_i transport was then measured at P_i concentrations of 1.19 mM and 2 mM. The control incubations contained 10 mM glucose and received a similar 40 min period of pre-incubation.

Protein estimation. The biuret method was used [11] with bovine serum albumin as standard.

Materials. Enzymes were obtained from Boehringer–Mannheim (Lewes, UK), and cell culture materials from ICN Flow, (High Wycombe, UK) or Gibco BRL (Uxbridge, UK). All other chemicals and biochemicals were obtained from Sigma or BDH (Poole, UK) except silicone oil that was purchased from Wacker Chemie (Walton-on-Thames, UK)

3. Results

Incubation of hepatocytes with dibutyryl cAMP for 15 min at 37°C results in an inhibition of the initial rate of P_i uptake (Fig. 1a). At a cAMP concentration of 10^{-7} M, the rate of P_i uptake is decreased by approxi-

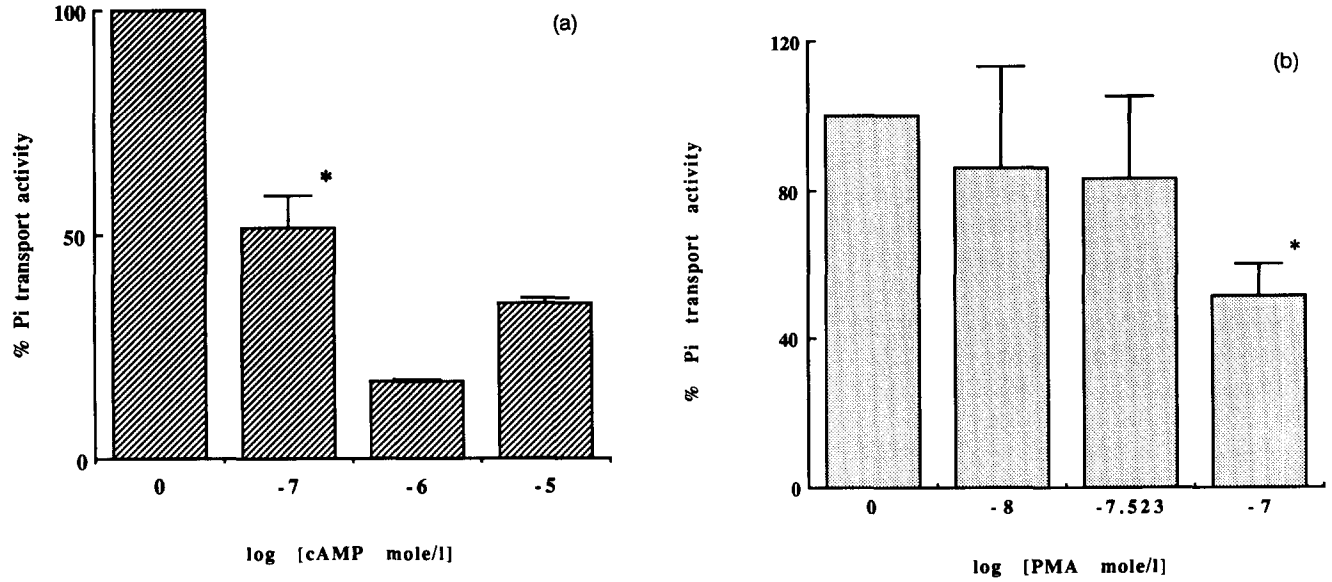

Fig. 1. Effect of dibutyryl cyclic AMP and phorbol ester on the initial rate of P_i uptake. Cells were pre-incubated at 37°C for 15 min in KRB containing P_i at 1.19 mM and the indicated concentrations of (a) cAMP or (b) PMA. The initial rate of P_i uptake was then determined following the addition of carrier-free [^{32}P]P_i to the princubation mixture. The results are the means plus S.E. of four experiments performed in duplicate with different batches of cells. *, Significantly different from control ($P < 0.02$).

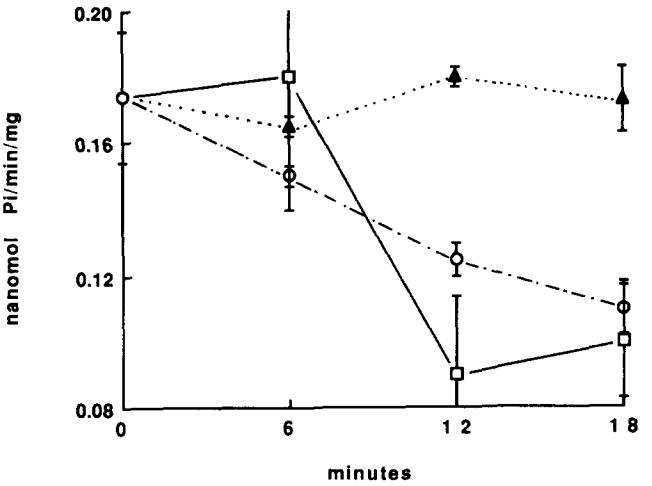

Fig. 2. Effect of incubation with cAMP and phorbol ester on P_i uptake by hepatocytes. The cells were preincubated for the indicated times with dibutyryl cAMP (○), PMA (□) or 4-α-phorbol (▲) at concentrations of 10^{-7} M before determination of the rate of P_i uptake. All data points are mean values obtained from three separate experiments performed in duplicate.

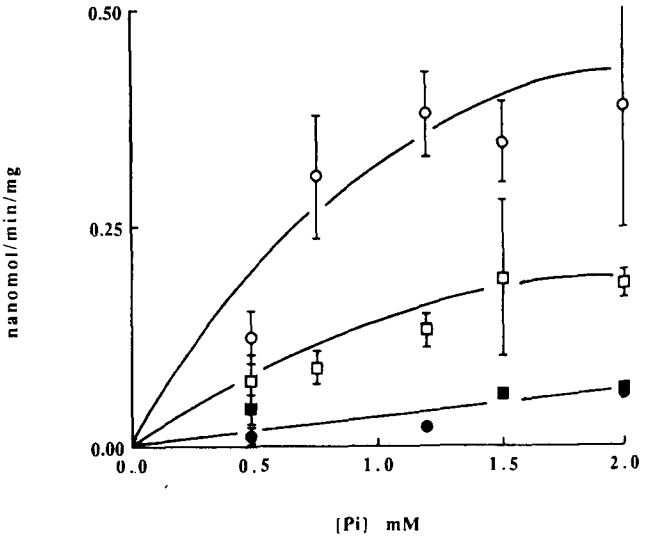

Fig. 3. Kinetics of P_i uptake by hepatocytes. The cells were incubated for 15 min with 10^{-7} M dibutyryl cAMP (□,■) or in KRB (○,●) before washing in P_i-free KRB and then suspension in KRB containing P_i at the indicated concentrations for determination of P_i uptake rate. In ■,●, Na^+ in the KRB was replaced by Li^+ ions. For the cAMP experiments the nucleotide was present throughout the washing steps and the transport measurements.

mately 50% ($P < 0.02$ relative to the control). Higher concentrations, up to 10^{-5} M, result in 70–80% inhibition of transport activity ($P < 0.01$ relative to the control). The effect of PMA concentration on P_i transport after 10 min of incubation is shown in Fig. 1b. No significant inhibition is observable unless the PMA concentration is at least 10^{-7} M.

The time dependencies of the inhibitions are shown in Fig. 2. Prolonged incubation with 10^{-7} M cAMP leads to a steady decrease in transport activity with a pseudo first-order rate constant of inactivation of $3.1 \cdot 10^{-2}$ min^{-1} calculated from data shown in the figure by a plot of $\ln[A_0/A_t]$ against time (not shown) where A_0 and A_t represent the transport rates at times 0 and t, respectively. The figure also contains results obtained for cells that were incubated with myristate, acetate phorbol ester (PMA) and 4-α-phorbol at concentrations of 10^{-7} M. PMA incubation is associated with a marked fall in transport activity that is time dependent, whereas the 4-α derivative is inert with respect to phosphate transport cabability.

To check that the inhibitory effect of cAMP was associated with the Na^+-dependent fraction of uptake, cells that had been incubated with cAMP for 15 min were used for measurements of the rate of P_i uptake at different P_i concentrations and the results compared with those obtained for cells similarly incubated in KRB alone. In other sets of experiments, cAMP-treated cells and non-treated controls were used for transport measurements at different P_i concentrations but transport was conducted in medium in which Li^+ ions replaced the Na^+ ions normally present in KRB. The results of these experiments are shown in Fig. 3. cAMP treatment brings about a decrease in Na^+ supported uptake at all P_i concentrations. Both treated cells and the controls exhibited a saturable uptake. Uptake in the presence of Li^+ was relatively slow and the relationship between transport rate and P_i concentration was linear. This uptake, assumed to be diffusional [4], was unaffected by prior treatment of the cells with cAMP.

After subtraction of the diffusion rates from the total uptakes, the results have been plotted in Fig. 4 in the form of $[P_i]/v$ against $[P_i]$ (Hanes plot) for determination of the transport kinetic parameters. Fitting the

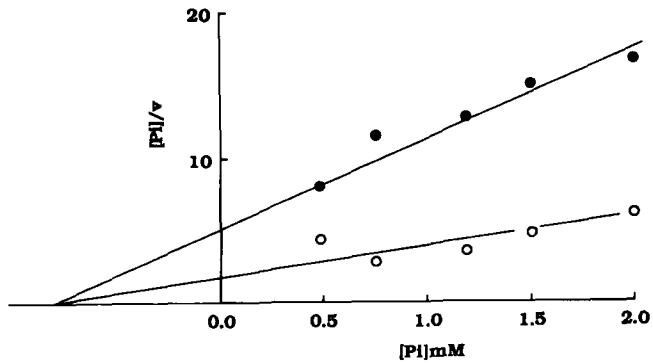

Fig. 4. Hanes S/v against S plot of the Na^+-dependent fraction of P_i uptake. The data points were extracted from those shown in Fig. 4 by subtracting the uptake values observed in the presence of Li^+ from the total uptake. ○, control; ●, cAMP-treated.

data to the Michaelis-Menten equation by weighted regression analysis [12] indicated K_m values of 0.77 ± 0.27 mM and 1.16 ± 0.22 mM for controls and cAMP treated cells, respectively. Equivalent V_{max} values are 0.52 ± 0.1 nmol P_i/min per mg protein and 0.23 ± 0.08 nmol P_i/min per mg. Thus incubation with cAMP produces a significant decrease in V_{max} of transport. The slight increase in K_m does not seem to be significant and the lines drawn to the data points in Fig. 4 assume that K_m is unchanged by cAMP.

Sodium-linked transport processes rely on an active Na^+K^+ATPase to establish a favourable gradient for Na^+ ions. Fructose is readily converted to fructose 1-phosphate by liver cells but subsequent metabolism is relatively slow because of the limited capacity of the fructose-1-phosphate aldolase. Fructose therefore tends to deplete hepatocytes of ATP and P_i [13–15]. The effect of fructose on Na^+-dependent P_i uptake was examined with cells that were provided with fructose as the repiratory substrate. The cells in primary culture were washed in KRB modified to contain 10 mM fructose instead of glucose and maintained in the fructose medium for 45 min before measurement of P_i uptake in the fructose-KRB. Cells harvested and incubated in glucose-KRB served as controls. The rate of P_i uptake was measured at 1.19 mM P_i and at 2 mM P_i. Uptake at P_i concentrations of 1.19 mM and 2 mM was inhibited by $56.7 \pm 6.0\%$ and $54.3 \pm 11.7\%$, respectively (data from 3 separate experiments performed in duplicate in each case). The inhibition is highly significant ($P < 0.005$) for both concentrations.

4. Discussion

The Na^+-P_i cotransporter of liver resembles that of the kidney proximal tubule in its sensitivity to cyclic AMP [7,8]; the maximum rate of transport is decreased but K_m is hardly affected. Whether the decrease in transport is a result of a fall in the number of tranporter molecules per unit of membrane or comes about by modification of the intrinsic carrier activity cannot be deduced from these experiments. Parathyroid hormone (PTH) seems to be the most important hormone for controlling P_i excretion, and cAMP production in response to hormone stimulation is assumed to play a significant role in the process by which the cotransporter is inhibited. Protein phosphorylation mediated by protein kinase A is therefore implicated [7]. PTH action may also involve phosphatidyl inositol turnover and therefore by implication, activation of protein kinase C [8].

In liver, cAMP is generated in response to glucagon and thus P_i uptake by liver cells is likely to be attenuated during gluconeogenic episodes. Since uptake is greatly stimulated by insulin [4], P_i transport into liver cells will be sensitive to the glucagon/insulin concentration ratio in the circulating blood. Glucagon stimulates glycogen breakdown and the phosphorylase reaction consumes P_i directly. Thus a transient fall in cytosolic P_i concentration could be expected to accompany the accelerated glycogen breakdown especially as uptake is inhibited. Any change in cytosolic P_i would be of short duration because enhanced activity of fructose-1,6-bisphosphatase 1 and of glucose-6-phosphatase under gluconeogenic conditions results in the re-release of P_i. Such considerations pass doubt perhaps on the notion that the cytosolic P_i concentration is an important determinant of transport activity in the short term.

Inhibition of P_i tranport after treatment of the hepatocytes with phorbol ester may be indicative of a role for protein kinase C in controlling P_i uptake by liver, but the relatively high concentrations of phorbol needed to affect transport, places some doubt on the physiological significance of these observations. It is possible that the phorbol treatment elevates the cytosolic Ca^{2+} concentration which then stimulates calmodulin-dependent protein kinase. The target(s) for the kinase could then be the same proteins as those phosphorylated by protein kinase A.

The experiments with fructose were an attempt to explore the connexion between cytosolic P_i concentration and transport capacity. The adjustment of P_i transport activity to P_i availability seems to be a general property of all cells that possess Na^+-P_i cotransport systems [7,8] but the molecular mechanisms by which the P_i signal is converted to altered transport activity are unknown. One candidate for the mechanism is a direct action of P_i on the transporter. Thus a fall of cytosolic P_i concentration accompanying fructose metabolism would be expected to stimulate transport by this model. In an early study of perfused liver [14], addition of fructose to the perfusion medium was reported to stimulate P_i uptake. Only inhibition of uptake was seen in our studies with isolated cells reported here however. Unfortunately, fructose depletes cellular ATP as well as P_i and therefore transport processes such as that for P_i which are dependent on the Na^+ gradient are affected indirectly by the fall in the cytosolic ATP. Our previous studies of P_i transport in liver [4] showed that treatment with 1 mM ouabain inhibits Na^+-dependent P_i transport by approximately 40% at an ambient P_i concentration of 1.19 mM. Also replacement of Na^+ ions by Li^+ ions inhibits cotransport at 2 mM P_i by a similar amount. Hence the observed 50–60% inhibition of Na^+-P_i cotransport in the presence of fructose probably could well derive from the depleted cellular ATP and mask any alteration in transport activity that may or may not have occurred in response to a lowered cytosolic P_i.

5. References

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