Biochimica et Biophysica Acta 856 (1986) 624–628
Elsevier

BBA 73054

The effect of gluconeogenesis on phospholipid turnover in isolated chick proximal tubule cells

Riffat Parveen, Michael F. Grahn and Peter J. Butterworth

Department of Biochemistry, King's College (Kensington Campus), Campden Hill Road, London W8 7AH (U.K.)

(Received October 8th, 1985)

Key words: Gluconeogenesis; Phospholipid turnover; Phosphate transport; (Chicken kidney)

Previous work from this laboratory has shown that isolated chick renal proximal tubule cells possess an Na^+-dependent P_i transport system and that P_i uptake is stimulated under gluconeogenic conditions. It is shown in the present paper that gluconeogenesis is associated with a rapid incorporation of P_i into membrane phospholipids, particularly phosphatidylinositol, and some evidence has been obtained for a change in the relative amounts of phosphatidylinositol polyphosphates under gluconeogenic conditions. There is no increase in the total phospholipid phosphate content however, suggesting that pyruvate-induced incorporation of P_i into phospholipids represents accelerated turnover rather than a net increase in synthesis. It is suggested that the stimulation of Na^+-dependent P_i uptake by pyruvate is related to the increased rate of phospholipid turnover. Thus P_i transport may be a further example of a physiological system that is influenced by phosphatidylinositol metabolism. The role of phosphatidylinositol phosphates could be to stimulate transfer of transporter molecules from internal stores to the brush-border membrane of the cell.

Introduction

Isolated proximal tubule cells from chick kidney take up P_i in a sodium-dependent process that is sensitive to inhibition by parathyroid hormone [1] and to stimulation by phosphate depletion [2]. In addition the cells are metabolically competent and actively produce glucose from pyruvate by gluconeogenesis [3]. As such they are suitable for studying biochemical events in the regulation of phosphate reabsorption by the kidney by various humoral agents.

The isolated cells from chick kidney take up P_i at a greater rate in the presence of a gluconeogenic substrate such as pyruvate than when supported on glucose as the respiratory substrate. Also when gluconeogenesis is blocked by the metabolic inhibitor 3-mercaptopicolinate [3], the stimulation of P_i uptake is also abolished [4]. The pyruvate-induced stimulation in P_i uptake occurs within two minutes, does not seem to originate from an elevated cellular ATP level, but may reflect in part a change in the intracellular organic phosphate/P_i ratio. The rapidity of the pyruvate effect suggests that a mechanism other than de novo synthesis of transporter molecules is responsible for the increase in Na^+-dependent P_i transport capacity.

There is growing interest in the role of phosphatidylinositol metabolism in the expression of the physiological effects of many hormones and neurotransmitters [5]. Parathyroid hormone has been demonstrated to increase the levels of phos-

Abbreviations: P_i, inorganic phosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

phatidic acid (PA), phosphatidylinositol (PI) and polyphosphoinositides in rabbit kidney cortex [6] and similar findings have been reported for rabbit kidney [7]. If PI metabolism is involved generally in regulating systems affecting phosphate reabsorption by the kidney, we considered the possibility that gluconeogenesis-induced potentiation of P_i uptake by chick kidney cells may also be accompanied by phospholipid metabolism. An investigation has been made therefore of membrane phospholipid turnover under conditions in which Na^+-dependent uptake of P_i is known to be stimulated.

Experimental

Isolated cells. Proximal tubule cells from Light Sussex-Rhode Island Red cross-bred chicks (Orchard Farms Ltd.) were prepared when the chicks were 3–6 weeks old. The isolation procedure was based on a published method [8] with some modifications. The medium was buffered with Tris at pH 7.4 and contained glucose (10 mmol/dm^3) as the respiratory substrate [8]. The concentrations of Ca^{2+} and P_i in the medium were 1.2 and 1.0 mmol/dm^3, respectively.

Collagenase (8 mg) and hyaluronidase (5 mg) were placed in a screw-capped glass vial containing 5 cm^3 medium. The vial and contents were kept at 37°C. Freshly removed chick kidneys were weighed and diced with scissors before transfer to the vial containing the enzymes. A further 5 cm^3 of medium (at 37°C) was added and digestion allowed to proceed while the contents of the vial were stirred continuously. After 15 min of treatment, the gas phase above the digest was replaced by O_2/CO_2 (95%/5%) to maintain aerobic conditions and aggregates of tissue were disrupted by passage through a pipette. The digestion was continued for a further 15 min before straining the suspension through nylon mesh (0.1 mm diameter) into a polythene tube. The volume was adjusted to 100 cm^3 with medium at 37°C and centrifuged at 1500 rpm in an M.S.E. Centaur 1 bench centrifuge for 30 s. The supernatant was discarded and the pellet resuspended in 100 cm^3 of medium and recentrifuged. This washing step was repeated twice more to ensure removal of adherent enzymes and contaminating cells such as erythrocytes. Finally the resultant pellet was suspended in medium to a protein concentration of 1 to 2 mg/cm^3 and maintained at 37°C.

Extraction of phospholipids. The cell pellet was homogenised with 10 vols. of chloroform/methanol (1 : 1, v/v) in a whirlimixer. A few glass beads were added to facilitate the homogenisation. All the organic phases containing phospholipids that accrued during the extraction procedure were mixed together and dried with a stream of nitrogen gas. The residue was redissolved in chloroform (300 μl) [9].

Incorporation of ^{32}P into phospholipids. Proximal tubule cells were suspended in medium (approx. 5 cm^3) at pH 7.4 containing glucose and equilibrated for 10 min at 37°C in closed glass vials in which the air space had been purged with O_2/CO_2 (95%/5%). An aliquot of a pyruvate solution was added to give a final concentration of 1 mmol/dm^3. Control incubations received a similar volume of medium. After 2 min of incubation in the presence of the added substrate [^{32}P]P_i was added to give a radioactivity of 1 μCi/cm^3 and incubation was continued for a further 10 min. The suspension was then washed twice by centrifugation in a bench centrifuge using 100 cm^3 of medium for each wash. The final pellet was then used for extraction for phospholipids.

Separation of phospholipids. Separation by chromatography involved the use of formaldehyde-treated paper [10]. Sheets (23 cm × 50 cm) of Whatman 1 paper were wrapped in a protective sheet of chromatography paper, tied gently with a cotton thread and soaked in 2.7 dm^3 of a mixture of 40% (w/v) formaldehyde and ethanoic acid (20 : 1, v/v) in a domestic pressure cooker. The contents were boiled for approx. 5 h. After cooling, the papers were washed under running tap water overnight. The washing was repeated at least five times with deionised water after which they were allowed to stand in deionised water for 4 h. The papers were then dried at room temperature (20°C).

The papers were used for descending chromatography with a solvent prepared as follows. A mixture of butan-1-ol/ethanoic acid/water (4 : 1 : 5, v/v) was equilibrated for 48 h with 0.125 vol. of diethyl ether. The upper layer of the mixture was used for chromatography. Samples were

applied in duplicate and chromatography conducted for 9–10 h at room temperature, after which the papers were removed and dried in a fume cupboard overnight. The dried papers were cut longitudinally into two equal halves. One half was stained by soaking in Nile blue solution for 30 min and then washed in running tap water for 1 min.

Areas containing resolved phospholipids were marked on the unstained half by reference to the stained spots on the other paper. The areas were cut out and cut into small pieces before transfer to scintillation vials containing 8 cm^3 of scintillation cocktail. The radioactivity incorporated into different phospholipids was determined in a Beckman LS 6300 scintillation spectrometer. The radioactivity remaining at the point of application on the chromatogram was also determined. Using known amounts of radioactivity, the recovery of activity was approx. 80% from chromatograms.

In other experiments phospholipids extracted under acid conditions as described by Schacht [11] were separated by thin-layer chromatography on aluminium sheets (20 cm × 20 cm) coated with silica gel 60 (0.2 mm) obtained from Merck Ltd., Darmstadt, F.R.G. The solvent system was chloroform/ethanol/3.3 N ammonia (43 : 38 : 12, v/v).

Total phosphate content of phospholipids. An aliquot (150 μl) of phospholipid extract was dried at 100°C. To the dried lipids 0.6 cm^3 of perchloric acid (60%) plus 0.2 cm^3 of water was added and the mixture was digested at 160°C for approx. 2–3 h until a clear solution was obtained. The clear solution was cooled to room temperature and then adjusted to 5 cm^3 with water before the addition of 0.5 cm^3 of 5% w/v ammonium molybdate plus 0.25 cm^3 of a solution containing 0.5 g 1-amino-4-sulphonic acid, 30 g sodium bisulphite and 6 g sodium sulphate in 250 cm^3 of water. After thorough mixing the solutions were allowed to stand for 30 min at 20°C before determination of the absorbance at 660 nm.

Protein. The protein content of cell preparations was determined by the method of Lowry et al. [12] using bovine serum albumin as a standard.

Results

After resolution by chromatography and staining, the phospholipid mixture extracted from chick proximal tubule cells appeared as dark-blue spots on a light-blue background but the individual components did not separate into sharp bands. There was no difficulty, however, in determining R_F values of resolved lipids for comparison with and identification from standard phospholipid markers. Phosphatidic acid had the highest R_F value, 0.82, followed by phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with R_F values of 0.78 and 0.76, respectively. Phosphatidylserine (PS) with an R_F of 0.56 and phosphatidylinositol (PI) with $R_F = 0.43$ were the slowest moving phospholipids. The migration rates were reproducible from separation to separation within a range of ±5%.

Pyruvate, when used as a respiratory substrate for the proximal tubule cell, brings about a considerable increase in the rate of P_i uptake by the cells [4]. Table I shows the incorporation of ^{32}P from [^{32}P]P_i into phospholipids when the cells were incubated with and without pyruvate. As indicated in Table I, there is a general enhancement of labelling of the phospholipids in the presence of pyruvate. The greatest increase appears in the PI fraction where labelling is 80% higher than in the control.

TABLE I

EFFECT OF PYRUVATE ON [^{32}P]P_i INCORPORATION INTO PHOSPHOLIPIDS OF CHICK PROXIMAL TUBULE CELLS

Cell suspensions (1–2 mg protein/cm^3) were pre-incubated at 37°C for 10 min before the addition of pyruvate (1 mmol/dm^3) to 'test' incubations. [^{32}P]P_i was added 2 min later and incubation continued for 10 min before separation of the cells and analysis of their phospholipids. The values shown are the mean ± S.E. of six different experiments performed in duplicate, each with a separate batch of cells.

Phospholipid	[^{32}P]P_i incorporated (cpm/20 μl extract)		
	control	pyruvate-treated	percentage stimulation
PI	416 ± 48	750 ± 43	80
PS	233 ± 17	378 ± 16	62
PA + PC + PE	785 ± 26	1342 ± 26	71

Because uptake of radioactive phosphate is increased in the presence of pyruvate [4], some of the raised level of radioactivity in the phospholipids could simply reflect normal turnover of phospholipid phosphate from cellular P_i that has acquired a higher specific radioactivity. To check whether the increased level of radioactivity does truly represent accelerated rate of turnover in the presence of pyruvate, renal cells were pre-equilibrated in the presence of [^{32}P]P_i before the addition of pyruvate to the incubation medium. The pre-incubation period of 10 min resulted in greater labelling of the phospholipids from an intracellular pool of radioactive P_i but, as shown in Table II, pyruvate stimulated the incorporation of label relative to control incubations. Tables I and II show that the several phospholipids tested all became labelled in these experiments but the labelling of PI was particularly noticeable both in the presence and absence of pyruvate. Considerable label was also incorporated into PA, PC and PE but the lack of separation of the fractions prevented an estimate of the labelling of each component.

The respiration rate increases from 3.64 to 4.7 nmol O_2/min per mg protein i.e. by 29%, when glucose is replaced by pyruvate as the respiratory substrate [4]. Assuming a P/O ratio of 3, ATP production would therefore be increased from 22 to 28 nmol/min per mg. This greater rate of ATP formation is likely to bring about a raised specific radioactivity of the cellular ATP pool and increased labelling of phospholipids during normal turnover of these compounds as a consequence. It is debatable however, whether the 29% change in ATP production can account for the observed 60–80% increase in the labelling of the phospholipids unless there is also some acceleration in the rate of turnover. When allowance is made for the amount of the extra ATP used for the production of glucose from pyruvate by these cells (estimated at 2.4 nmol ATP/min per mg [4] under these conditions), it is not unreasonable to conclude that the metabolic turnover of phospholipipds is accelerated in the presence of pyruvate.

TABLE II

STIMULATION OF [^{32}P]P_i INCORPORATION INTO PHOSPHOLIPIDS OF TUBULE CELLS PRE-EQUILIBRATED WITH [^{32}P]P_i

Cell suspensions were pre-equilibrated at 37°C with [^{32}P]P_i for 10 min before the addition of pyruvate (2 mmol/dm^3) to 'test' incubations. After 2 min the cells were separated for analysis of phospholipids. The values shown are mean ± S.E. of four determinations in duplicate, each with different batches of cells.

Phospholipid	[^{32}P]P_i incorporated (cpm/20 μl extract)		
	control	pyruvate-treated	percentage stimulation
PI	3130 ± 66	5511 ± 49	76
PS	493 ± 67	575 ± 72	17
PA + PC + PE	2954 ± 42	4404 ± 33	49

TABLE III

EFFECT OF PYRUVATE ON [^{32}P]P_i INCORPORATION INTO PHOSPHOLIPIDS OF TUBULE CELLS

Cell suspensions were pre-incubated at 37°C for 10 min before the addition of pyruvate. [^{32}P]P_i was added 2 min later and after incubation for a further 10 min, the cells were separated, phospholipids were extracted and then analysed by thin-layer chromatography [11].

	[^{32}P]P_i incorporated (cpm)				
	PIP_2	PIP	PI	PA	PC
Control cells	166	41	42	33	104
Pyruvate-treated cells	130	77	52	57	97

Table III shows the result of a single experiment in which certain of the phospholipids were separated by thin-layer chromatography. The results of this single experiment must be interpreted cautiously but there is some suggestion that pyruvate may stimulate a change in the ratio of PIP and PIP_2.

The total phosphate content of phospholipids extracted from pyruvate-treated cells was indistinguishable from that found for non-treated controls. The values obtained were 7.56 ± 0.95 μg/100 mg phospholipid and 7.57 ± 0.89 μg/100 mg for controls and pyruvate-treated respectively ($n = 24$ in each case). These results imply that the observed stimulation in ^{32}P-labelling of phospholipids in the presence of pyruvate is not accompanied by a net increase in phospholipid synthesis de novo.

Discussion

The gluconeogenic substrate pyruvate consistently increased P_i incorporation into phospholipids. The stimulation of incorporation into PI, however, was greater than into other phospholipids of the renal proximal tubule cells. The experiments in which cells were preequilibrated with [^{32}P]P_i before application of the pyruvate stimulus revealed that labelling of PS was relatively slight. Since this phospholipid is outside the phosphatidate-polyphosphoinositide cycle it is not surprising that PS remains relatively unlabelled if PI is the prime target for the labelled phosphate. The enhanced radioactivity measured collectively in PE, PC and PA may represent labelling of PA predominantly if the phosphatidate polyphosphoinositide cycle is stimulated under gluconeogenic conditions. Cortical tubules from rat kidney have been shown to incorporate [^{32}P]P_i into PI, PE and PC in the presence of lactate [13,14] but the greatest degree of incorporation was into PI.

Stimulation of ^{32}P incorporation into the phospholipids of chick renal cells was evident after incubating the cells with pyruvate for only 2 min. This rapid onset of stimulation compares well with previous reports which indicate that PI turnover is usually very rapid. The time of incorporation ranges from 2 s in blood platelets to a few minutes in other tissues [5].

In many tissues application of a humoral stimulus results in an accelerated rate of turnover of PI. The increased turnover is thought to occur by a cycle of reactions linking PA and polyphosphoinositides and the incorporation of phosphate [5]. Parathyroid hormone and cyclic 3′,5′-AMP increase the levels of PA, PI and polyphosphoinositide in rabbit kidney cortex [6,7] and the regulatory effect of this hormone on phosphate reabsorption by the proximal tubules of the kidney may require PI metabolism for its full expression.

When isolated chick proximal tubule cells and the renal epithelial cell line LLC-PK_1 are depleted of phosphate there is a stimulation of Na^+-dependent Pi uptake into these cells which is demonstrable within 30–60 min of the onset of the depletion [2,15]. The stimulation models the adaptation that occurs in whole animal studies of phosphate depletion where P_i excretion becomes considerably reduced. One explanation for the rapid onset is that the increased phosphate transport capacity results from a transfer of transporter molecules from an intracellular pool to a functional state in the brush-border membrane. A similar argument has been advanced to account for part of the stimulation of P_i uptake into chick proximal tubule cells under gluconeogenic conditions [4]. If membrane traffic is an important agent in the regulation of phosphate transport by stimuli such as parathyroid hormone, dietary restriction of phosphate and gluconeogenesis, phosphatidyl phosphate metabolism may play a part in initiating the recycling of the Na^+-dependent phosphate transporter.

Acknowledgments

We thank the Wellcome Trust for their support of this work and for providing a salary to M.F.G. and the Government of Pakistan and the British Council for a grant to R.P.

References

1 Parveen, R., Grahn, M.F. and Butterworth, P.J. (1984) FEBS Lett. 176, 220–222
2 Grahn, M.F., Parveen, R. and Butterworth, P.J. (1985) Abstract No. 41. 7th International Workshop on Phosphate and Other Minerals, Mineral Electrolyte Metab. 11, 12
3 Robinson, B.K. and Oei, J. (1975) FEBS Lett. 58, 12–15
4 Grahn, M.F., Parveen, R. and Butterworth, P.J. (1985) Cell Biochem. Function 3, 193–198
5 Berridge, M.J. (1984) Biochem. J. 220, 345–360
6 Farese, R.V., Bidot-Lopez, P., Sabir, A., Smith, J.S., Schinbeckler, B. and Larson, R. (1980) J. Clin. Invest. 65, 1523–1526
7 Khalifa, S., Mills, S. and Hruska, K.A. (1983) J. Biol. Chem. 258, 14400–14406
8 Larkins, R.G., MacAuley, S.J., Rapoport, A., Martin, T.J., Tulloch, B.R., Byfield, P.G.H., Matthews, E.W. and MacIntyre, I. (1974) Clin. Sci. Mol. Med. 46, 569–582
9 Michell, R.H., Hawthorne, J.N., Coleman, R. and Karnovsky, M.L. (1970) Biochim. Biophys. Acta 210, 86–91
10 Kai, M. and Hawthorne, J.N. (1966) Biochem. J. 98, 62–67
11 Schacht, J. (1981) Methods Enzymol. 72, 626–632
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Wirthensohn, G., Lefrank, S., Wirthensohn, K. and Guder, W. (1984) Biochim. Biophys. Acta 795, 392–400
14 Wirthensohn, G., Lefrank, S. and Guder, W. (1984) Biochim. Biophys. Acta 795, 401–410
15 Caverzasio, J., Brown, C.D.A., Biber, J., Bonjour, J.-P. and Murer, H. (1985) Am. J. Physiol. 248, F122–F127