ABSENT PYRUVATE INHIBITION OF PYRUVATE DEHYDROGENASE KINASE IN LACTATING RAT MAMMARY GLAND FOLLOWING VARIOUS TREATMENTS

Removal of circulating insulin and prolactin and exposure to protein synthesis inhibitors

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1. Introduction

In several mammalian tissues, including the rat mammary gland, the mitochondrial PDH complex is inactivated by phosphorylation catalysed by an intrinsic $MgATP^{2-}$-requiring kinase and activated by dephosphorylation catalysed by an intrinsic Mg^{2+}/Ca^{2+}-dependent phosphatase [1–4]. We have reported [5,6] that in lactating rat mammary gland 24 h starvation leads to a change in the behaviour of PDH kinase which persists in whole tissue or mitochondrial extracts and is unlikely to be due to changes in relative concentrations of effector pairs (ATP/ADP, acetyl CoA/CoA and NADH/NAD) shown to operate on the analogous enzyme in other tissues [1,7,8]. The PDH kinase from lactating mammary gland of starved rats is more active than that from glands of fed animals under apparently identical conditions in vitro and loses the property of being inhibited by pyruvate.

Similar phenomena have subsequently been reported for PDH kinase of hearts taken from starved or diabetic rats [9]. We have now extended our experiments to include situations where the lactating rats have been deprived of insulin or prolactin for 3 h and 24 h, respectively. Earlier data [10] showed large increases in phosphorylation of mammary gland PDH under these conditions. Since both diabetes and starvation are associated with inhibition of protein synthesis [11,12] we investigated whether inhibition of protein synthesis by other means might have similar effects. This has proven to be the case. We have also shown that changes in PDH kinase behaviour are rapidly reversible in vivo by large doses of insulin. Other experiments have shown that the variable inhibition of PDH kinase by pyruvate demonstrable in vitro can be of significance in vivo and that if a new effector is involved it must be very tightly bound to the complex.

Abbreviations PDH, pyruvate dehydrogenase; DTT, dithiothreitol, i.p., intra-peritoneal, s.c., sub-cutaneous; IU, international unit, EDTA, ethylene diamine tetra-acetate; EGTA, ethanedeoxybis (ethylamine)-tetra-acetate

2. Experimental

2.1. *Materials*

Sources of rats and chemicals were the same as in [4,6,10] with the following additions. Cycloheximide and puromycin (dihydrochloride) were obtained from Sigma London, Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH. Dichloracetic acid was obtained from BDH Chemicals Ltd, Poole, Dorset BH12 4NN.

2.2. *Methods*

The procedures used for freeze-clamping mammary gland, for preparing and extracting mammary gland mitochondria, assaying plasma glucose and assaying PDH activity and protein in tissue or mitochondrial extracts were as in [4,6,10].

Injections were given while rats were minimally anaesthetized with ether. Times of injection in relation to freeze-clamping or removal of glands are

stated in the tables. Control experiments showed that injection of 0.9% saline, at 0 5 h or 3 h before freeze-clamping did not affect 'initial' or 'total' PDH activities (defined below).

'Initial activity' of PDH in tissue extracts was determined following extraction into medium containing 5 mM EDTA. 'Total activity' was the maximal activity of PDH exhibited in other extracts not containing EDTA to which 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ and pig heart PDH phosphatase (4 U/ml) were added before 5–10 min incubation at 30°C. 'Percentage total activity' of PDH in any extract referred to in tables or text is the ratio of 'initial' to 'total' PDH activity multiplied by 100.

Mitochondria were extracted by sonication into a medium containing 30 mM Triethanolamine, 1 mM DTT (pH 7), Lubrol (0.1% w/v) and rat serum (5% v/v) [final conc. 17–20 mg protein/ml]. Following complete activation of PDH by Mg^{2+}, Ca^{2+} and excess PDH phosphatase as described above, 5 mM EGTA and 5 mM ATP with or without 1 mM sodium pyruvate were added and the incubation continued. Samples were removed at intervals to determine the time course of inactivation. These time courses were used to compare the susceptibility to inhibition by pyruvate of PDH kinase in the extracts. This comparison was facilitated by calculating a quantity referred to as the 'fractional pyruvate inhibition' which is explained below. The fall in activity of PDH incubated with ATP reflects increasing phosphorylation of the complex except at the extremes of phosphorylation or dephosphorylation [13]. Consequently this fall in activity of PDH during a given time of incubation is a rough index of average PDH kinase activity. Over short time courses as in the experiments shown in fig.1 the presence of EGTA prevents interference by PDH phosphatase. If the PDH activity in an extract before exposure to ATP, after exposure to ATP and after exposure to ATP and pyruvate are symbolized by a, a_1, a_2 respectively then the 'fractional pyruvate inhibition' is:

$$\frac{(a-a_1)-(a-a_2)}{(a-a_1)}$$

When $a_1 = a_2$ the value of this expression is zero and there is no pyruvate inhibition. When $a = a_2$ the value of the expression is 1 and pyruvate has completely inhibited PDH kinase activity. Intermediate degrees of inhibition are indicated by values between 0 and 1.

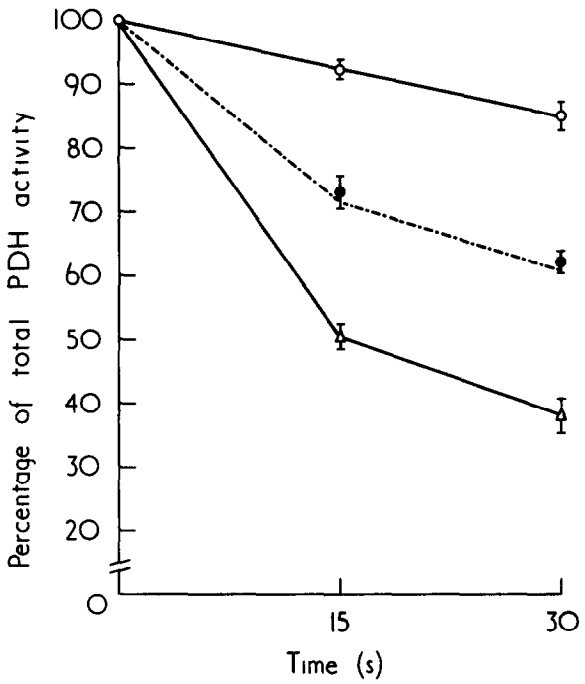

Fig.1. Inhibition of PDH activity due to 5 mM ATP in the presence of 1 mM pyruvate in extracts of mammary gland mitochondria taken from fed or starved rats. Mitochondrial preparation and the procedure for determining time course of ATP inactivation are in section 2. Total PDH activity in these incubations was 1.85 ± 0 12 units/ml. Vertical bars indicate SEM. (o——o) Extracts from fed rats each point based on 5–9 separate preparations. (△——△) Extracts from starved rats each point based on 10–13 separate preparations. (●,●) Data from mixture of extracts from fed and starved rats contributing equal concentrations of PDH activity each point based on 4 such mixtures. (—. —. —) Line connecting calculated means of the data from independently incubated extracts of fed and starved rats.

3. Results and discussion

Table 1, rows 1–3, columns 3, 4, includes published data which is helpful for comparison with new data in rows 4–9. Deprivation of circulating prolactin for 24 h by injection of 2-bromo-α-ergocryptine [14] or of insulin for 3 h by streptozotocin injection [15] led to large reductions in percentage total activity of PDH

Table 1
The effect of various treatments of lactating rats on inhibition by pyruvate of PDH kinase activity in extracts of mammary glands

	Treatment of animal	Plasma glucose (mM)	Percentage total activity of PDH	Fractional pyruvate inhibition
1	Control fed, no injections	7.0 ± 0.8 (8)	39.1 ± 1.7 (9)	0.70 ± 0.01 (6)
2	Starved 24 h	6.3[b] ± 0.6 (7)	10.2 ± 0.5[e] (11)	0.01 ± 0.01[e] (4)
3	Starved 24 h; insulin (10 IU) injected i.p., 0.5 h	2.1[g] ± 0.4[e] (9)	27 1[g] ± 1.7[d] (7)	0 77[g] ± 0.06 (3)
4	Fed Streptozotocin (65 mg/kg) injected i.p , 3 h	18 4 ± 2 6[d] (8)	5.7 ± 1.1[e] (5)	0.07 ± 0.03 (6)
5	Fed Streptozotocin (65 mg/kg) injected i.p., 3 h and insulin[a] (10 IU) injected i.p., 1 h	3.5[g] ± 0 4[d] (4)	33.5[g] ± 2.1[c] (4)	0.50[g] ± 0.06[c] (4)
6	Fed· 2-bromo-α-ergocryptine (1 mg) injected s.c , 24 h	9 9 ± 1.7 (5)	21.3 ± 3.0[e] (11)	0.07 ± 0 04[e] (4)
7	Fed· Cycloheximide (2 mg/kg) injected i.p., 2 h	6.9 ± 0.2 (4)	21.0 ± 1.8[e] (8)	0.06 ± 0.04[e] (5)
8	Fed Cycloheximide (2 mg/kg) injected i.p , 2 h and insulin[a] (10 IU) injected i.p., 1 h	4.9[g] ± 0.1[c] (4)	41.8[g] ± 3.2 (4)	–
9	Fed· Puromycin (50 mg/kg) injected i.p., 2 h	9.4 ± 0.6[c] (4)	18.7 ± 3 2[e] (4)	

[a] Glucose, 6 nmol, injected s.c at the same time as the insulin injection in order to maintain blood glucose
[b] Samples only from 24-h starved animals
[c] $p<0.05$, [d] $p<0.01$, [e] $p<0.001$ for differences from control rats according to the '*t*' test
[f] $p<0.01$, [g] $p<0.001$ for differences of data in rows 3 compared to 2, 5 compared to 4 and 8 compared to 7 according to the '*t*' test

Percentage total activity of PDH (defined in section 2) was determined on extracts of glands freeze-clamped while the animals were under halothane/O_2 anaesthesia. The 'fractional pyruvate inhibition' of PDH kinase (defined in section 2) was determined on mitochondrial extracts and based on 30 s incubation with 5 mM ATP ± 1 mM pyruvate. Total PDH activity in the gland extracts was 11.4 ± 0.3 munits/mg protein and 542 ± 19 munits/ml and in the mitochondrial extracts was 56.5 ± 1.9 munits/mg protein and 1628 ± 65 munits/ml. None of these parameters differed between experimental groups. Plasma glucose was determined on blood samples obtained from neck veins of the animals immediately after removal of the glands. Data are means ± SEM with no. observations in parentheses

in freeze-clamped gland (rows 6,4). A similar effect on PDH activity was seen following injections of cycloheximide (row 7) and puromycin (row 9) in doses reported to inhibit maximally cytosolic protein synthesis in rat liver and other tissues [16–18]. None of these treatments led to observable differences in behaviour of the animals in comparison with fed controls. Suckling by the pups continued to the time of anaesthetic induction. During the period of these experiments, 9 a.m.–12 noon, rats feed very little even when food is available. In fact, withdrawal of food for this period was without effect on mammary gland PDH (% total activity of PDH 39.1 ± 1.1 [3] – c.f. row 1 of table 1). Consequently, it is unlikely that reduced food intake or reduced suckling contributes to the effect of streptozotocin, cycloheximide or puromycin. Treatment with streptozotocin, cycloheximide and 2-bromo-α-ergocryptine led to loss of the inhibition by 1 mM pyruvate of extracted PDH kinase activity (column 5) which may contribute to the increased steady state level of phosphorylation of the PDH complex in vivo which was also induced by these treatments. Both inhibition of PDH kinase by pyruvate and the level of pyruvate dehydrogenase phosphorylation appropriate to fed untreated animals were largely restored within 1 h by injections of insulin into starved or diabetic animals (rows 3,5). Further evidence that inhibition of PDH kinase by pyruvate is relevant to control of the activity of PDH complex in vivo is shown in table 2. Injection of pyruvate into fed but not starved animals increased within 30 min the % total activity of PDH in freeze-clamped mammary gland. A small effect was seen when dichloracetate instead of pyruvate was injected

Table 2
Percentage total activity of PDH in extracts of freeze-clamped mammary glands from fed and starved rats injected with sodium pyruvate or sodium dichloracetate

Treatment of rat		Injection	Time (min) of injection before freeze-clamping	Percentage total activity of PDH
1	Fed	None		39.1 ± 1.7 (9)
2	Fed	Pyruvate	30	54.7 ± 1 9[a] (4)
3	Fed	Pyruvate	60	40.1 ± 4.6 (4)
4	Fed	Dichloracetate	60	79.8 ± 3 6[a] (4)
5	24 h starved	None		10.2 ± 0.5 (11)
6	24 h starved	Pyruvate	30	9.4 ± 0.8 (4)
7	24 h starved	Dichloracetate	60	17.7[b] ± 2.5 (4)

[a] $p<0$ 001 for differences of data in rows 2, 3, 4 from data in row 1 according to the 't' test

[b] $p<0.01$ for difference of data in rows 6 and 7 from data in row 5 according to the 't' test

Sodium pyruvate or sodium dichloracetate was injected i.p. at a dosage of 1 μmol/g wt of rat. Procedures for freeze-clamping and assay are in section 2. Total PDH activity in these extracts was 12.7 ± 0.4 (munits/mg protein) and was unaffected by the treatments Data are means ± SEM with no observations in parentheses

into starved animals (row 7). This matches in vitro experiments with the enzyme complex in mitochondrial extracts from starved animals where a weak inhibition of PDH kinase was shown by 1 mM dichloracetate whereas 1 mM pyruvate was totally ineffective (results not shown). It has been reported that the PDH kinase from heart is more powerfully inhibited by dichloracetate than by pyruvate [19].

The mechanism whereby these various treatments influence behaviour of PDH kinase can only be speculative at the present time. Our conclusion at this stage is that pyruvate inhibition of PDH kinase is a variable factor which actually is relevant to the control of PDH activity in vivo.

Experiments shown in fig.1 were designed to detect an easily diffusible factor in extracts of mitochondria from fed or starved animals which might underlie the difference in susceptibility of PDH kinase to inhibition by pyruvate. The time course of ATP inactivation of PDH in the presence of 1 mM pyruvate was determined in mixtures of mitochondrial extracts from fed and starved animals in which volumes of both extracts in the mixtures were adjusted such that each would make an equal contribution to the total PDH activity. Clearly, the time course of PDH inactivation in such a mixture was exactly what would be predicted if the PDH complexes in each extract behaved completely independently. Furthermore, the difference in susceptibility to pyruvate inhibition of PDH kinase in mitochondrial extracts from fed and starved animals was not abolished in the enzyme complexes obtained by isoelectric precipitation (pH 5.6) from such extracts and resuspended in fresh extraction medium to final conc. 0.7–2 units/ml total PDH activity In these latter experiments we avoided the use of high concentrations of $MgCl_2$ during the activation of mammary gland PDH by exogenous PDH phosphatase. PDH phosphatase, $MgCl_2$ and $CaCl_2$ (final conc. 4 units/ml, 0.04 mM and 0.1 mM, respectively) were added to the resuspended isoelectric precipitates. Incubation of the mixture for 15 min at 30°C was sufficient to activate fully the PDH in the precipitates. Fractional pyruvate inhibition of the PDH kinase was then determined during 5 min of further incubation as explained in section 2 Values of this expression were 0.69 ± 0.10 [4] for the PDH complex from fed animals and 0 02 ± 0.01 [4] for the PDH complex from starved animals.

Modifications of PDH kinase behaviour which persist when the PDH enzyme complex is highly diluted and partially purified suggest a covalent change in the PDH kinase or its substrate, the

α-subunit of the complex. However, it is still possible that a very tightly bound effector could be present after these procedures.

No simple explanation is available for the effects of cycloheximide and puromycin even if one assumes that their only direct action is to inhibit protein synthesis. If continuous synthesis of a polypeptide were required to maintain pyruvate inhibition of PDH kinase it is not expected that insulin injection could reverse the effect of cycloheximide treatment on PDH kinase. Furthermore, other experiments (data not shown) indicate that insulin injection into starved rats can restore the normal level of mammary gland PDH phosphorylation in spite of a previous injection of cycloheximide. It is conceivable that cycloheximide and puromycin treatment could diminish circulating prolactin and thus mimic the effect of 2-bromo-α-ergocryptine. It was found earlier [10] that insulin injection can counteract the effect of 2-bromo-α-ergocryptine injection on the percentage total activity of mammary gland PDH.

Further purification of PDH complexes from fed, starved and cycloheximide-treated animals will be required to determine what changes in the complexes are involved in the alterations in behaviour which we have described.

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