Effect of phenylephrine on pyruvate dehydrogenase activity in rat hepatocytes and its interaction with insulin and glucagon

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In isolated rat hepatocytes phenylephrine promotes a rapid increase in the amount of pyruvate dehydrogenase present in its active form (PDH_a). This action is mediated by α_1-adrenergic receptors and is not observed in Ca^{2+}-depleted hepatocytes. It is mimicked by the Ca^{2+} ionophore A23187. No changes in metabolites known to affect PDH activity are measured 3 min after addition of phenylephrine. Glucagon also increases PDH_a, its action is additive to that of phenylephrine. The action of phenylephrine on PDH_a could be explained by an increase in mitochondrial free Ca^{2+}.

(Liver) *Pyruvate dehydrogenase* *α_1-Adrenergic receptor* *Glucagon* *Ca^{2+}* *Ionophore A23187*

1. INTRODUCTION

The administration of vasopressin to the perfused rat liver leads to a rapid increase in the amount of pyruvate dehydrogenase (PDH) present in its active, non-phosphorylated, form [1]. The same effect has been observed in isolated hepatocytes [2,3] in which it was found that angiotensin and phenylephrine activate pyruvate dehydrogenase in a similar manner. The amount of mammalian pyruvate dehydrogenase complex present in its active, non-phosphorylated form is determined by the relative activities of its interconverting phosphatase and kinase, each of which is controlled by various effectors [4,5]. The phosphatase is activated by Mg^{2+} ($K_{0.5} \simeq 1$ mM, where $K_{0.5}$ is the concentration required for half-maximal effect) and by Ca^{2+} ($K_{0.5} \simeq 1\ \mu M$). The Mg–ATP-dependent kinase is inhibited by pyruvate, ADP and perhaps Ca^{2+} and activated by increases in the ratios of NADH/NAD and acetyl CoA/CoA (see [5] for review).

Vasopressin, angiotensin and α_1-adrenergic agents are thought to activate phosphorylase kinase by increasing the concentration of Ca^{2+} in the cytosol of liver cells [6–9]. However, the origin of this calcium is still under debate.

Among the mechanisms which could explain the activation of PDH by vasopressin, an increase in mitochondrial Ca^{2+} would be the most likely, since measurements of whole tissue concentration of metabolites known to affect PDH kinase or phosphatase could not explain the activation of the enzyme [1]. This postulated increase in mitochondrial Ca^{2+} is however in contradiction with measurements of Ca^{2+} in mitochondria prepared from hepatocytes [10,11] or perfused liver [12] after addition of vasopressin or phenylephrine.

This study was undertaken to explore the possibility that the α-adrenergic activation of pyruvate dehydrogenase is accomplished by an increase in mitochondrial Ca^{2+}, by trying to answer the following questions:

(i) Do α-adrenergic agonists modify PDH activity through α_1 (mediating Ca^{2+} changes) or α_2 receptors?
(ii) Is this action Ca^{2+}-dependent?

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(iii) Can the increase in PDH activity be due to changes in concentrations of metabolites known to modify the activity of PDH kinase or phosphatase?
(iv) How do α-adrenergic agents interact with the action of insulin and glucagon on PDH activity?

2. MATERIALS AND METHODS

Hepatocytes from fed 250 g male SIVZ (a Wistar-derived strain) rats were prepared by collagenase digestion [13,14]. They were preincubated and incubated in a Krebs-Ringer bicarbonate buffer containing 3% dialyzed bovine albumin (fraction V Sigma) and 10 mM glucose. For calcium-free experiments hepatocytes were preincubated and incubated in Krebs-Ringer bicarbonate buffer containing no calcium and 0.5 mM EGTA. Cell viability estimated by trypan blue exclusion was >92%.

After the incubation, aliquots (1 ml) of cells were centrifuged (10 s), the supernatant removed and the pellet frozen in liquid nitrogen for subsequent determination of pyruvate dehydrogenase activity or ATP, ADP, pyruvate, acetoacetate, β-hydroxybutyrate and coenzyme A. Another aliquot of cell plus medium was immediately frozen and later thawed in 5 vol. of 100 mM NaF/20 mM EDTA/0.5% glycogen in a 100 mM glycylglycine buffer (pH 7.4). Phosphorylase *a* activity was determined as in [15,16].

For the determination of pyruvate dehydrogenase activity, the frozen cell pellets (100 mg wet wt/ml) were extracted at 0°C by disruption (at least 10 passages) in a 1 ml syringe fitted with a 0.6 mm diam. needle in 100 mM KH_2PO_4 (pH 7.3), 2 mM EDTA/1 mM dithiothreitol/0.1% Triton X-100 which contained 50 μl/ml of rat serum to prevent proteolysis [17], and then frozen and thawed 3-times. The suspension was then thawed and spun for 30 s in a centrifuge (Eppendorf 3200), and the supernatant was assayed for pyruvate dehydrogenase activity [18]. This activity is referred to as 'initial activity' (PDH_a). No activity was sedimented with the pellet fraction. One unit of pyruvate dehydrogenase activity is that amount which catalyses the transformation of pyruvate at the rate of 1 μmol/min at 30°C.

For measurements of oxygen consumption, hepatocytes (25 mg/ml) were pre-equilibrated at 37°C with 95% O_2/5% CO_2 to reach 60–70% oxygen saturation and transferred to the oxygen chamber (Rank Brother Co., Cambridge). Basal oxygen consumption was recorded for 5–7 min before the addition of hormone. The absolute values of oxygen consumption under control conditions varied from one cell preparation to another: 1.66 ± 0.11 μmol $O_2 . min^{-1} . g^{-1}$ ($n = 4$). Since phenylephrine increased oxygen consumption by only 20–30% the agonist effect was expressed in percent change *vs* control. For the determination of ATP, ADP, pyruvate, acetoacetate, β-(−)hydroxybutyrate and coenzyme A, the cell pellet was homogenized in 1 M $HClO_4$, then centrifuged, the supernatant was neutralized and the metabolites measured as in [19].

Except where stated, results are given as mean ± SEM for a given number of different cell preparations. Each value used to calculate the mean was derived from triplicate incubations. The data are expressed/g wet wt of hepatocytes.

Phenylephrine bitartrate was from Sigma. Regular Insulin U-40 was obtained from E.L. Lilly (Indianapolis IN), glucagon from Novo, Denmark. Prazozin was a gift from Pfizer AG, Belgium. Yohimbine was from C. Roth (Karlsruhe), all the enzymes, coenzymes were from Boehringer (Mannheim).

3. RESULTS

3.1. *Activation of PDH by phenylephrine and by the Ca^{2+} ionophore A23187*

Phenylephrine promotes a rapid increase in the amount of active, non-phosphorylated pyruvate dehydrogenase (PDH_a) (fig.1). The action is maximal after 1 min. Shorter time points have not been measured (the error on time introduced by the 10 s centrifugation would be too large). The effect on PDH_a lasts at least 20 min. When the hepatocytes are preincubated and incubated in a Ca^{2+}-free medium containing 0.5 mM EGTA, they do not respond anymore to phenylephrine by an increase in PDH_a (table 1). A slight, but significant increase in phosphorylase activity is still observed (table 1). Addition of the Ca^{2+} ionophore A23187 to hepatocytes incubated in a buffer containing Ca^{2+} is followed by a rapid and long-lasting increase in

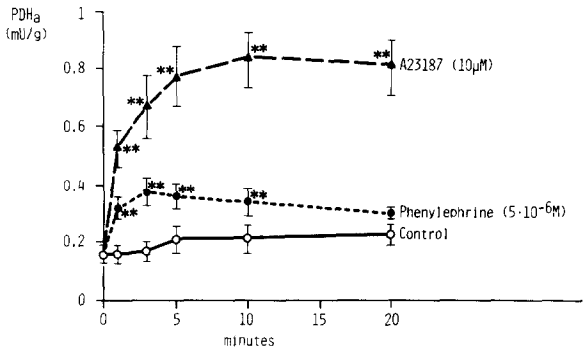

Fig.1. Effect of phenylephrine and of the Ca^{2+} ionophore A23187 on 'initial' pyruvate dehydrogenase activity in isolated hepatocytes from fed rats. ** $p \leqslant 0.005$ using paired Student's t-test.

PDH_a. This increase is not observed when Ca^{2+} is omitted from the medium (not shown). The dose-dependence for phenylephrine of PDH activation is quite similar to that of phosphorylase activation (fig.2).

3.2. *Suppression of the effect of phenylephrine on PDH activity and oxygen consumption by α_1-adrenergic blockers*

Table 1 shows that the α_1-antagonist prazozin at a concentration inhibiting phosphorylase activation by adrenergic agents also suppresses the phenylephrine effects in the activation of PDH and the stimulation of oxygen consumption, whereas the α_2 antagonist, yohimbine, does not modify these actions.

3.3. *Absence of action of phenylephrine on ATP/ADP, pyruvate, coenzyme A and mitochondrial NADH/NAD ratio*

Phenylephrine increases fructose 2,6-bisphosphate in hepatocytes [20] and thereby may increase glycolysis and pyruvate concentration. Since PDH activity can be modified by several metabolites (among which pyruvate), or coenzymes, their level was measured in isolated hepatocytes 3 min after the addition of phenylephrine, a time at which the effect of the agonist on PDH is maximal. Table 2 shows that neither ATP, ADP, pyruvate, coen-

Table 1

Effect of phenylephrine on phosphorylase, on 'initial' pyruvate dehydrogenase activity and on oxygen consumption in normal and Ca^{2+}-depleted hepatocytes: addition of prazozin and yohimbine

Addition	Phosphorylase (units/g)	PDH (munits/g)	O_2 consumption (% of control)
Normal hepatocytes			
Control	6.2 ± 0.9	0.15 ± 0.03	
Prazozin 10^{-6} M	7.4 ± 1.5	0.16 ± 0.03	
Yohimbine 5×10^{-6} M	7.3 ± 1.8	0.15 ± 0.02	
Phenylephrine 5×10^{-6} M	17.8 ± 0.9[a]	0.28 ± 0.03[a]	127 ± 1[a]
Phenylephrine + prazozin	7.6 ± 1.9[b]	0.18 ± 0.02[b]	96 ± 2[b]
Phenylephrine + yohimbine	17.0 ± 1.4[a]	0.26 ± 0.03[a]	124 ± 6
Ca^{2+}-depleted hepatocytes			
Control	2.2 ± 0.2	0.09 ± 0.01	
Phenylephrine 5×10^{-6} M	5.9 ± 0.2[a]	0.07 ± 0.01	106 ± 3

[a] Significantly different from control: $p \leqslant 0.025$ using paired Student's t-test
[b] Significantly different from phenylephrine: $p \leqslant 0.25$ using paired Student's t-test

Hepatocytes were incubated as described in section 2. Phosphorylase and PDH activities were measured 3 min after the addition of phenylephrine. Absolute basal values for oxygen consumption were: control, 1.66 ± 0.11; yohimbine, 1.64 ± 0.17; prazozin, 2.25 ± 0.35 μmol $O_2 \cdot min^{-1} \cdot g^{-1}$; values are means ± SEM of 3–4 hepatocyte preparations

Table 2

Absence of effect of phenylephrine on ATP, ADP, pyruvate, Coenzyme A and mitochondrial NADH/NAD ratio

	ATP (μmol/g)	ADP (μmol/g)	Pyruvate (nmol/g)	*B/A*	Coenzyme A (nmol/g)
Control	3.14 ± 0.15	0.88 ± 0.09	174 ± 29	1.55 ± 0.11	103 ± 12
Phenylephrine 5×10^{-6} M	3.01 ± 0.05	1.00 ± 0.11	189 ± 38	1.59 ± 0.13	103 ± 7

B/A, β-hydroxybutyrate/acetoacetate ratio

Hepatocytes were incubated as described in section 2; 3 min after addition of NaCl or phenylephrine, cells were rapidly centrifuged and the pellet frozen in liquid N_2; values are means ± SEM of 3 hepatocyte preparations

zyme A, nor mitochondrial NADH/NAD ratio as measured by the β-hydroxybutyrate/acetoacetate ratio, were altered 3 min after phenylephrine addition.

3.4. *Interaction of phenylephrine with the action of insulin and glucagon on PDH activity*

Insulin is able to antagonize the action of phenylephrine on phosphorylase activity [21,22]. Insulin is also able to increase PDH_a but to a lesser extent than phenylephrine [23] (table 3). When insulin is added together with phenylephrine, no significant change in PDH_a is observed compared to phenylephrine alone, whereas in the same experiments insulin has significantly reduced the activity of phosphorylase activated by phenylephrine (phenylephrine alone, 22.1 ± 1.05; phenylephrine + insulin, 19.9 ± 1.04 units/g, $p \leqslant 0.005$ using paired Student's *t*-test). Glucagon promotes a very marked increase in PDH_a (table 3). When phenylephrine and glucagon are added together at concentrations giving maximal response, their action on PDH_a is additive. It is also long-lasting since 20 min after the addition of the two hor-

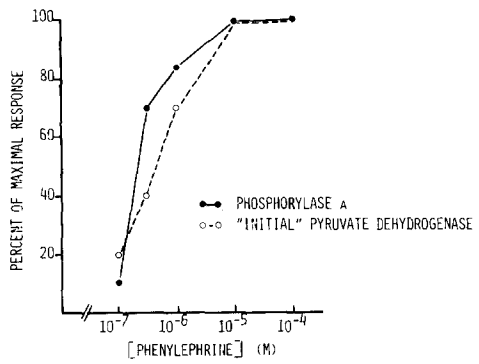

Fig.2. Sensitivity of phosphorylase and of 'initial' pyruvate dehydrogenase to phenylephrine in isolated hepatocytes from fed rats. The data are expressed as percentages of maximal response of each of the enzymes to phenylephrine. Results are from 2 different hepatocyte preparations which gave similar results.

Table 3

Interaction of phenylephrine with the action of insulin and glucagon on 'initial' pyruvate dehydrogenase activity in hepatocytes from fed rats

Addition	PDH_a	
	3 min	20 min
Control	0.40 ± 0.04	0.41 ± 0.06
Phenylephrine 5×10^{-6} M	0.64 ± 0.10[a]	0.57 ± 0.06[a]
Glucagon 10^{-9} M	0.93 ± 0.11[a]	0.65 ± 0.06[a]
Phenylephrine + glucagon	1.23 ± 0.08[a,b]	0.97 ± 0.12[a,b]
Insulin 1 munit/ml	0.56 ± 0.10	0.61 ± 0.07[a]
Phenylephrine + insulin	0.64 ± 0.08[a]	0.65 ± 0.07

[a] Significantly different from control: $p \leqslant 0.025$ using paired Student's *t*-test

[b] Significantly different from glucagon alone: $p \leqslant 0.025$ using paired Student's *t*-test

Hepatocytes were incubated as described in section 2; values are means ± SEM of 4–6 hepatocyte preparations

mones, PDH_a is still higher than with either hormone alone.

4. DISCUSSION

Phenylephrine rapidly increases 'initial' pyruvate dehydrogenase activity. This action is not observed when hepatocytes are Ca^{2+}-depleted and it is blocked by the α_1-blocker prazozin whereas the α_2-adrenergic blocker yohimbine is without effect. The Ca^{2+} ionophore A23187 is also able to promote an increase in PDH_a. This agent may however act either via an increase in cytosolic Ca^{2+}, followed by an increase in mitochondrial Ca^{2+} or by an increase in fructose 2,6-bisphosphate [20] and glycolysis. This would lead to an increase in pyruvate which could inhibit pyruvate dehydrogenase kinase.

The action of phenylephrine cannot be explained by changes in whole cell concentration of ATP, ADP, pyruvate, coenzyme A or in the mitochondrial redox state as measured by β-hydroxybutyrate–acetoacetate ratio since none of them is modified at a time when phenylephrine increases PDH_a.

Insulin has no effect on phenylephrine-stimulated PDH, whereas in the same experiments it does slightly, but significantly, decrease phosphorylase *a*. If the two hormones were acting on PDH via the same mechanism one would expect an additivity or a potentialisation of their action. Since the mechanism of action of insulin on phenylephrine-stimulated phosphorylase is not known (e.g., decrease in the amount of Ca^{2+} mobilized, modification in the sensitivity of phosphorylase kinase to Ca^{2+}) no hypothesis can be made on the lack of interrelation of the two hormones on PDH_a. The most unexpected data are obtained with glucagon alone or with phenylephrine; glucagon by itself increases markedly the activity of PDH. Such an action was not found in vivo [2] 30 min after injection of the hormone. When phenylephrine is added with glucagon both at a concentration giving maximal effect, a further increase in PDH_a is measured (table 3) and this increase lasts at least 20 min. This suggests that the two agents do not act on the enzyme by the same mechanism. It does not exclude that the same activator is involved and that the action of glucagon and of phenylephrine on this activator are additive. Recent data [24] have shown that glucagon, in the presence of vasopressin, is able to increase calcium content in rat liver mitochondria. Since the additivity of glucagon and phenylephrine on PDH_a is also observed with vasopressin (not shown), such a mechanism could explain the marked and sustained increase in PDH activity. The problem, however, remains open if we consider the action of phenylephrine alone. In many studies [10–12], a decreased total mitochondrial Ca^{2+} has been measured in organelles isolated from phenylephrine or vasopressin-treated liver or hepatocytes. Phenylephrine has also been shown to rapidly decrease free Ca^{2+} in rat liver mitochondria [25]. On the other hand, calcium-dependent increases in several mitochondrial functions have been described after addition of α-adrenergic agents, vasopressin or angiotensin. The 3 agents increased transiently NADH fluorescence before increasing oxygen consumption [26]. Glucagon had the same action. Addition of EGTA suppressed the action of all the hormones on NADH fluorescence [26]. Vasopressin also caused a decrease in concentration of 2-oxoglutarate in hepatocytes from fed rats [27]. This decrease was not observed in the absence of Ca^{2+} and is consistent with a Ca^{2+}-dependent activation of 2-oxoglutarate dehydrogenase [27].

In conclusion, we have shown that phenylephrine increases PDH_a in hepatocytes from fed rats. This action is Ca^{2+}-dependent and could reflect an increase in mitochondrial Ca^{2+}.

The present data emphasize the discrepancy between the decreased mitochondrial Ca^{2+} content measured after α-adrenergic agents or vasopressin, and the indirect evidence of the Ca^{2+}-dependent changes in mitochondrial function (among which an increase in PDH_a).

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