Decreased flux through pyruvate dehydrogenase during calcium ion movements induced by vasopressin, α -adrenergic agonists and the ionophore A23187 in perfused rat liver

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Vasopressin or α -adrenergic agents such as phenylephrine or adrenaline, but not glucagon, elicited an initial decrease in flux through pyruvate dehydrogenase assayed by $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate in perfused rat liver. This rapid decrease in $^{14}\text{CO}_2$ production was maximal within 1–2 min of exposure, concomitant with a rise in effluent pyruvate concentration: a subsequent return towards initial values in both parameters was completed well before 5 min. This time course was superposed with Ca^{2+} efflux from perfused liver, maximal (at 116 nmol/min per g wet wt. of liver) at 1–2 min of exposure. The percentage of the active (dephospho) form of pyruvate dehydrogenase was not decreased at 2 min of exposure. The effect on flux through pyruvate dehydrogenase by phenylephrine was abolished by prazosine, phentolamine or phenoxybenzamine. Ionophore A23187 also caused a depression in $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate and a rise in effluent pyruvate concentration, but this effect was stable for longer times, and it was delayed when Ca^{2+} was omitted from the perfusion medium. Responses of phenylephrine and A23187 were not additive. The results demonstrate that under the experimental conditions employed in intact perfused liver, the mitochondrial multienzyme system of pyruvate dehydrogenase is sensitive to vasopressin, α -adrenergic agents and A23187. The similar time course in Ca^{2+} efflux may be indicative of the involvement of Ca^{2+} in mediating this effect.

The effects of α -adrenergic agonists and hormones such as vasopressin in promoting hepatic glycogenolysis have been attributed to an elevation in $[\text{Ca}^{2+}]$, leading to an activation of extramitochondrial glycogen phosphorylase *b* kinase (for reviews, see Exton, 1980; Williamson *et al.*, 1981). The rise in cytosolic $[\text{Ca}^{2+}]$ is associated with a loss of mitochondrial Ca^{2+} (Babcock *et al.*, 1979; Blackmore *et al.*, 1979) and also of a pool located close to the surface of the cell (Althaus-Salzmann *et al.*, 1980). Although a transient rise in the cytosolic free concentration of Ca^{2+} has been shown (Murphy *et al.*, 1980), the time course of the mitochondrial free Ca^{2+} concentration under the influence of α -adrenergic agonists is less clear.

Denton & McCormack (1980) suggested that the mitochondrial matrix free Ca^{2+} concentration is a dynamic parameter with its set-point near the sensitive region of key intramitochondrial dehydrogenases, 0.1–10 μM . One of these enzymes is pyruvate dehydrogenase, with the Ca^{2+} -sensitive

component being pyruvate dehydrogenase phosphate phosphatase, as shown for heart, kidney and white fat (Denton *et al.*, 1972) and liver (Roche & Cate, 1977). With perfused liver, Hems *et al.* (1978) have shown, in fact, that upon addition of vasopressin there is an activation of pyruvate dehydrogenase, and it was suggested that this is brought about by an increase in the mitochondrial Ca^{2+} concentration.

The time course of Ca^{2+} movements upon hormonal stimulation is such that a peak efflux from perfused liver (Blackmore *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Sies *et al.*, 1981; Reinhart *et al.*, 1982a) and a rise in cytosolic $[\text{Ca}^{2+}]$ in isolated hepatocytes (Murphy *et al.*, 1980) is reached after about 1–2 min, and these are completed well before 5 min. Since the first sample taken by Hems *et al.* (1978) was at this later point in time, we decided to assess the time course of the activation of pyruvate dehydrogenase in perfused rat liver more closely. Interestingly, we observed an overlap of a

decrease and an increase in flux through this reaction, these being distinguishable by an appropriate choice of initial pyruvate concentration. An initial decrease in flux through pyruvate dehydrogenase upon α -adrenergic stimulation forms the focus of the present paper. Determination of the percentage of the active (dephospho) form showed that this initial change in flux does not correspond to a decrease in the steady-state content of the active form.

Materials and methods

Livers from male Wistar rats (160–200 g body wt.), fed on stock diet (Altromin, Lage, Germany) were perfused at 37°C without recirculation of the perfusate (Sies, 1978), using bicarbonate-buffered salt solution equilibrated with O₂/CO₂ (19:1, v/v) (Krebs & Henseleit, 1932). Unless stated otherwise, the medium also contained L-lactate (2.1 mM) and pyruvate (1 mM) as sodium salts. Perfusate flow (4–5 ml/min per g wet wt.) was maintained constant throughout individual experiments. Ca²⁺ activity in the effluent perfusate was measured with a Ca²⁺-sensitive electrode (IS561-Ca²⁺; Philips, Kassel, Germany) and an R44/2-SD/1 reference electrode filled with 1 M-KNO₃, operated as described previously (Sies, 1976; Sies *et al.*, 1981). In view of the small changes observed in the μ M range on the basal level of 1.25 mM-Ca²⁺ in the perfusate, high amplification plus a bucking voltage were required. Perfusate O₂ concentration and pH were also monitored using electrodes, and effluent O₂ concentration was maintained >0.2 mM to avoid pericentral hypoxia. Production of ¹⁴CO₂ from [1-¹⁴C]pyruvate (Häussinger *et al.*, 1975) and the concentrations of metabolites in the effluent perfusate (cf. procedures described in Bergmeyer, 1974) were determined as described previously.

For measurement of the tissue content of pyruvate dehydrogenase, samples of freeze-stopped liver tissue (400–500 mg) were homogenized in 10 vol. of potassium phosphate buffer (50 mM), pH 7.0, containing 0.2% Lubrol PX and 1% rat serum. Homogenization was carried out at 0°C with a tissue disintegrator (Ultraturrax TP 10; Jahnke and Kunkel, Staufen, Germany) for two 30 s periods with a 30 s interval, and centrifuged in an Eppendorf centrifuge (model 3200) at room temperature for 2 min. Pyruvate dehydrogenase activity in the supernatants was determined by coupling to the arylamine acetyltransferase reaction by a modification of the methods described by Wieland *et al.* (1972) and Walajtys *et al.* (1974). Arylamine acetyltransferase was prepared from pigeon liver acetone-dried powder by the method of Tabor *et al.* (1953), except that the step of adsorption to alumina gel was omitted. The assay system was exactly as

that used by Walajtys *et al.* (1974), except that cysteine (5 mM) was used as the sulphydryl reagent. The reaction was started by the addition of pyruvate (10 mM) and the decrease in absorbance at 405 nm was followed at 25°C. For blanks, pyruvate was omitted. Total pyruvate dehydrogenase activity was obtained after incubation of liver extracts with MgCl₂ (20 mM) for 60 min at 25°C, before centrifugation (Wieland *et al.*, 1972; Walajtys *et al.*, 1974; Patel *et al.*, 1982).

Chemicals and biochemicals were obtained from Boehringer (Mannheim, Germany), Merck (Darmstadt, Germany) or Sigma Chemical Co. (München, Germany) except for ionophore A23187, which was from Calbiochem (Giessen, Germany), prazosin, a gift from Pfizer (Karlsruhe, Germany), phenoxybenzamine, a gift from Röhm Pharma (Weiterstadt, Germany), phentolamine, a gift from Ciba-Geigy (Wehr, Germany), and verapamil, a gift from Knoll (Ludwigshafen, Germany).

Results

Vasopressin and α -adrenergic agonist effects on ¹⁴CO₂ production from [1-¹⁴C]pyruvate

Fig. 1(a) shows that ¹⁴CO₂ production from [1-¹⁴C]pyruvate by perfused liver is increased upon infusion of vasopressin towards a new steady state, in agreement with the observation of Hems *et al.* (1978), who found that the proportion of pyruvate dehydrogenase present in its active form is substantially increased in rat liver within 5 min of perfusion with vasopressin. However, there is an initial decrease in ¹⁴CO₂ production before the increase sets in, occurring within 1–2 min of exposure to vasopressin (Fig. 1a). The initial decrease and the subsequent increase can be separated by prior activation of pyruvate dehydrogenase. As shown in Fig. 1(b), there is only an initial decrease when the steady-state activity is increased beforehand by raising the pyruvate concentration in the perfusate from 50 μ M to 1 mM. The time of appearance of the initial decrease coincides closely with the initial peak of glucose release (Fig. 1) and other parameters described below. The effect on flux through pyruvate dehydrogenase is not seen when glucagon is infused instead of vasopressin (Fig. 2), although the effect on glucose release is even more pronounced.

Observations similar to those with vasopressin were obtained with several α -adrenergic agonists, including phenylephrine (Fig. 3) or adrenaline (Fig. 4). In the latter case, the effect on O₂ uptake by the perfused liver is also shown, which again exhibits a clear separation of a faster first phase from a more delayed second phase, as observed previously by others (Scholz & Schwabe, 1980). Interestingly,

Fig. 1. Responses of $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ -pyruvate and glucose output from isolated perfused rat liver upon addition of vasopressin

The influent pyruvate concentration was 0.05 mM (a) or 1.0 mM (b). The time points assayed for pyruvate dehydrogenase activity by Hems *et al.* (1978) are indicated by ● in (a).

Fig. 2. Effects of glucagon and vasopressin on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate and glucose release from perfused liver

Fig. 3. Effect of phenylephrine on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate and glucose release and its abolition in the presence of an α -adrenergic antagonist prazosin. Additions were made during the times indicated in the panels at the top of the Figure.

Sugano *et al.* (1980) observed a transient reduction of nicotinamide nucleotides during the first phase.

The responses of $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate and of glucose release, as well as of other parameters not shown here, were abolished by appropriate α -adrenergic antagonists such as prazosin (Fig. 3), phentolamine or phenoxybenzamine at similar concentrations (results not shown). Verapamil had no effect.

The dose-response curve for the phenylephrine effect on $^{14}\text{CO}_2$ production from $[1-^{14}\text{C}]$ pyruvate is shown in Fig. 5, where the difference in the rates

between the lowest level after phenylephrine addition and the subsequent steady state is plotted for individual perfusion experiments. Half-maximal concentration is slightly above $0.1 \mu\text{M}$ -phenylephrine, similar to other responses such as that found for phosphorylase *a* (Blackmore *et al.*, 1978). Fig. 6 demonstrates the capability of a single perfused liver to respond repetitively to the α -adrenergic agonist.

Effluent pyruvate concentrations exhibit a transient rise after the hormonal stimulation (Fig. 7), in

Fig. 4. Effect of adrenaline on $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and O_2 uptake by perfused liver

Fig. 5. Dose-response curve of the phenylephrine response

The difference in the rates between the lowest level upon exposure to phenylephrine and the subsequent steady state was plotted for each individual perfusion experiment, represented by a single point. Compare the results in Fig. 3.

accordance with the inhibition of flux through pyruvate dehydrogenase.

Ca^{2+} efflux

As mentioned in the introduction, previous work on perfused liver has shown that the peak of Ca^{2+} efflux is reached after about 1–2 min. Since those investigations (Blackmore *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Sies *et al.*, 1981; Reinhart *et al.*, 1982a) were carried out at low extracellular

Fig. 6. Repetitivity of phenylephrine response. Maximal effects (compare the results in Fig. 5) can be obtained sequentially with one perfused liver.

Fig. 7. Effect of phenylephrine on effluent pyruvate concentration

Ca^{2+} concentrations (μM range), Ca^{2+} efflux was examined also at the physiological Ca^{2+} concentration of 1.25 mM used in the perfusion medium. Vasopressin, as well as phenylephrine, elicits an efflux of about $24 \mu\text{M}\text{-Ca}^{2+}$ under these conditions (Fig. 8), amounting to a peak rate of $116 \pm 5 \text{ nmol of } \text{Ca}^{2+}/\text{min per g wet wt.}$ ($n = 4$). Clearly, the Ca^{2+} movements observed in the extracellular space are superposable with the first fast phase of the metabolic responses shown above. Re-uptake of Ca^{2+} from the medium after cessation of hormone infusion is more pronounced with phenylephrine (Fig. 8b) than with

Fig. 8. Effects of vasopressin (a) and phenylephrine (b) on effluent Ca²⁺ concentration

Caval perfusate was passed through an electrode chamber (Sies, 1976, 1978) containing a Ca²⁺-sensitive electrode and a reference electrode. Owing to the high medium concentration of 1.25 mM-Ca²⁺, amplification was high and resulted in elevated noise. Calibrations of signal with (shown in b) or without the perfused liver present were identical.

vasopressin, but these slight differences in Ca²⁺ movements were not studied in more detail in the present paper.

Response to ionophore A23187

Ionophore A23187, like the α-adrenergic agonists, is known to release Ca²⁺ from an exchangeable pool of hepatocytes (Chen *et al.*, 1978; Blackmore *et al.*, 1978). Ionophore A23187 is capable of eliciting a decrease in ¹⁴CO₂ release from [1-¹⁴C]pyruvate (Fig. 9a) concomitant with the known increase in glucose production (not shown) and an increase in effluent pyruvate concentration (Fig. 9b), similar to the observations with the α-adrenergic agents. However, this effect is stable for a longer period of time (compare Fig. 9 with Figs. 3 and 7). Interestingly, the effects of ionophore A23187 and phenylephrine are not additive. This is shown in Fig.

Fig. 9. Effect of ionophore A23187 on ¹⁴CO₂ production from [1-¹⁴C]pyruvate (a) and on effluent pyruvate concentration (b)

In (b), the further addition of phenylephrine is shown to result in a negligible further effect on effluent pyruvate concentration.

Table 1. Non-additivity of A23187 and phenylephrine effects on ¹⁴CO₂ production from [1-¹⁴C]pyruvate

Data are from an experiment of the type shown in Fig. 9(b). Control refers to steady state before additions; other data are for lowest (peak) values obtained.

	¹⁴ CO ₂ production from [1- ¹⁴ C]pyruvate (μmol/min per g wet wt.)
Control	0.93
A23187 (4.9 μM)	0.75
A23187 (4.9 μM) plus phenylephrine (1.3 μM)	0.75

Table 2. *Pyruvate dehydrogenase (active form) in perfused rat liver under the influence of vasopressin and phenylephrine*
Livers were perfused with influent lactate (2.1 mM) and pyruvate (1 mM) as in Fig. 1(b). Consecutive tissue samples were taken and freeze-clamped at the times indicated. Addition of hormones was from 36 min on. Pyruvate dehydrogenase activity content in the samples was determined as described in the Materials and methods section and is expressed in terms of m-units/g of liver wet wt. and as % of total. Results are means $\pm$ S.E.M. for the numbers of different perfusion experiments shown in parentheses.

Additions (at 36 min)	Pyruvate dehydrogenase present in active form at:					
	35 min		38 min		44 min	
	(m-units/g of liver)	(% of total)	(m-units/g of liver)	(% of total)	(m-units/g of liver)	(% of total)
None	254 $\pm$ 13 (3)	44 $\pm$ 2 (3)	269 $\pm$ 17 (3)	44 $\pm$ 4 (3)	284 $\pm$ 23 (3)	43 $\pm$ 4 (3)
Vasopressin (12 nM)	239 $\pm$ 11 (9)	48 $\pm$ 3 (9)	310 $\pm$ 22 (5)	57 $\pm$ 4 (5)	412 $\pm$ 17 (5)	70 $\pm$ 3 (5)
Phenylephrine (1 μ M)			232 $\pm$ 22 (4)	46 $\pm$ 3 (4)	260 $\pm$ 5 (4)	47 $\pm$ 2 (4)

9(b) for the effluent pyruvate concentration and for the flux through pyruvate dehydrogenase shown in Table 1. When Ca^{2+} was omitted from the perfusion medium, the effect of the ionophore was delayed (results not shown).

Effects on the steady state of the active (dephospho) form of pyruvate dehydrogenase

The active form and the total content of pyruvate dehydrogenase were assayed at 35 min of perfusion and, after starting the infusion of vasopressin or phenylephrine at 36 min, further samples of liver tissue were taken at 38 and 44 min, in order to evaluate whether the early and late effects on flux through pyruvate dehydrogenase and Ca^{2+} release are reflected by changes in the active form of the enzyme. In perfusions under the conditions of Fig. 1(a) (low pyruvate), the initial percentage of the active form was 29% of the total, and it increased to 59% at 2 min of exposure to vasopressin (14 nM) and remained at this level 5 min later (means of two experiments). Thus, the findings of Hems *et al.* (1978) are reproduced and extended to the earlier time point of 2 min of exposure. Further measurements were carried out under the conditions of Fig. 1(b) [high pyruvate (1 mM)], the results being shown in Table 2. When no addition was made, the percentage of the active form remained at about 44%, with the total activity tending to increase slightly as the consecutive samples were taken. With both vasopressin (12 nM) and phenylephrine (1 μ M) there was no decrease at 2 min of exposure; in fact, with vasopressin there was already a slight increase in the percentage of the active form of pyruvate dehydrogenase at this time point, an effect not seen with phenylephrine.

Thus the early effects of vasopressin and the α -adrenergic agonists on flux through pyruvate dehydrogenase are not accompanied by a decrease in the steady-state content of the active (dephospho) form of the enzyme. Similarly, A23187 (5 μ M) gave

no change at 2 min and also not at 5 min of exposure (three experiments). However, when the perfusion medium was changed from high pyruvate (1 mM) to low pyruvate (0.05 mM) with no other additions made, the percentage active form decreased from 56% to 39% within 2 min (two experiments). Furthermore, when an infusion of dichloroacetate (2.7 mM) in the presence of low pyruvate (0.05 mM) was terminated at 36 min of perfusion, the active form of the enzyme decreased from 96% to 44% within 2 min (two experiments) thus indicating that a short-term decrease in the content of the active form can be ascertained by the method employed.

Discussion

Initial decrease of flux through pyruvate dehydrogenase

The present work demonstrates that upon exposure of perfused rat liver to vasopressin or to α -adrenergic agonists there is an initial decrease in $^{14}\text{CO}_2$ production from [1- ^{14}C]pyruvate, followed by a return to higher flux (Figs. 1–4). This effect is attributed to an initial decrease in flux through pyruvate dehydrogenase, as supported by a rise in effluent pyruvate concentration that follows a similar time course (Fig. 7), whereas the effect on glucose release is divided into a faster first phase that parallels the above-mentioned responses, and an extended second phase that terminates only after the infusion of the hormone is stopped (Figs. 1 and 3).

Although theoretically an increase in the rate of flux through the pyruvate carboxylase reaction might also lead to a decrease in $^{14}\text{CO}_2$ production, a significant contribution of this reaction to the observed decrease in $^{14}\text{CO}_2$ production appears unlikely. As shown in Fig. 2, glucagon has no effect on $^{14}\text{CO}_2$ production, but both glucagon and α -adrenergic agonists were shown to stimulate

pyruvate carboxylase flux (Garrison & Borland, 1979), so that if present, an effect of pyruvate carboxylase on the $^{14}\text{CO}_2$ production observed here is negligible.

The hormonal effects on flux through pyruvate dehydrogenase as seen with phenylephrine and other α -adrenergic agonists are likely to be mediated by α -receptors, because there is no effect in the presence of appropriate antagonists such as prazosin (Fig. 3). Thus a general (non-specific) effect seems to be ruled out.

Relationship to Ca^{2+} movements

The time course of the decrease of flux through pyruvate dehydrogenase parallels the efflux of Ca^{2+} into the effluent perfusate (Fig. 8). These Ca^{2+} movements occur at a peak rate of 116 nmol/min per g wet wt., and they are in phase with the cytosolic increase in free Ca^{2+} as studied with isolated hepatocytes (Murphy *et al.*, 1980). However, the possibility that the amount of the active (dephospho) form of pyruvate dehydrogenase in the intact cell may be controlled by the free Ca^{2+} concentration in the mitochondrial matrix (Denton & McCormack, 1980) is not borne out by the present observations, as shown in Table 2. Several factors are involved in regulation of both the subcellular Ca^{2+} concentrations (see Carafoli *et al.*, 1982) and the activity of and flux through the pyruvate dehydrogenase complex (see e.g. Patel *et al.*, 1982). Ca^{2+} could be involved in mediating the initial decrease of flux through pyruvate dehydrogenase, in line with the observation of decreased flux upon addition of ionophore A23187 (Fig. 9), which is known to release Ca^{2+} from mitochondria. Interestingly, the state of phosphorylation of the enzyme was also not influenced by A23187.

Responses in nicotinamide nucleotide reduction upon α -adrenergic stimulation (Sugano *et al.*, 1980) occur concomitantly with the Ca^{2+} movements and could explain the effect on flux through pyruvate dehydrogenase without affecting the amount of active enzyme present. In this regard, it is noteworthy that the flux through pyruvate dehydrogenase as assayed by $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$ pyruvate in the presence of NH_4Cl was substantially less with β -hydroxybutyrate than with acetoacetate present in the medium (Häussinger *et al.*, 1975). Thus, it will be of interest to determine whether the effect observed by Sugano *et al.* (1980) is selectively mitochondrial rather than cytosolic. In experiments with isolated hepatocytes, Yip & Lardy (1981) showed that Ca^{2+} caused a decrease in the lactate/pyruvate ratio and an increase in the β -hydroxybutyrate/acetoacetate ratio, and concluded this to indicate a stimulation of transfer of reducing equivalents from cytosol to mitochondria.

Alternative explanations for the present observa-

tions that do not require changes in mitochondrial matrix free Ca^{2+} concentrations should also be considered. For example, Davis & Martin (1982) reported α -adrenergic-dependent lowering of the membrane potential across the mitochondrial inner membrane in fat-cells, and Kessar & Crompton (1981) observed changes in Ca^{2+} -cycling in heart mitochondria upon addition of α -adrenergic agents; however, both these effects were stable and, if at all, would apply to the second phase rather than the first phase, which was the focus of the present study.

Since this paper was submitted, the work of Reinhart *et al.* (1982b) on Ca^{2+} fluxes in perfused liver appeared, showing results similar to the observations in Fig. 8. Furthermore, the Ca^{2+} content of mitochondria (fraction 8) isolated from phenylephrine-treated liver was found to be decreased from 2.7 to 1.9 nmol/mg of protein within 90 s of exposure. Interestingly, work by Coll *et al.* (1982) that was published recently indicated that the activity coefficient of mitochondrial matrix Ca^{2+} decreased 2-fold upon phenylephrine addition to perfused liver, so that it can be concluded from the combined results that the matrix free Ca^{2+} decreased from approx. $2.7\mu\text{M}$ to $0.8\mu\text{M}$, assuming that the relationship of 10 nmol/mg of protein corresponding to about $10\mu\text{M}$ - Ca^{2+} (Coll *et al.*, 1982) can be extrapolated to lower values. Thus, free Ca^{2+} concentration may be below saturation for some mitochondrial processes in liver.

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