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ENERGY-LINKED REGULATION OF GLUCOSE AND PYRUVATE OXIDATION IN ISOLATED PERFUSED RAT HEART

ROLE OF PYRUVATE DEHYDROGENASE

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SUMMARY

1. The regulation of glycolysis and pyruvate oxidation under varying conditions of ATP and oxygen consumption was studied in isolated perfused rat hearts. Potassium-induced arrest was employed to inhibit the ATP consumption of the heart.

2. Under the experimental conditions, the beating heart used solely glucose as the oxidisable substrate. The glycolytic flux through the aldolase step decreased in pace with the decreasing oxygen consumption during the potassium-induced arrest of the heart. The decrease in glucose oxidation was larger than the inhibition of the oxygen consumption, suggesting that the arrested heart switches to fatty acid oxidation.

The time course and percentage changes of the inhibition of pyruvate oxidation and the decrease in the amount of the active form of pyruvate dehydrogenase suggest that the amount of active pyruvate dehydrogenase is the main regulator of pyruvate oxidation in the perfused heart.

3. To test the relative significance of the possible mechanisms regulating covalent interconversions of pyruvate dehydrogenase, the following parameters were measured in response to the potassium-induced cardiac arrest: concentrations of pyruvate, acetyl-CoA, CoA-SH, citrate, α-oxoglutarate, ATP, ADP, AMP, creatine, creatine phosphate and inorganic phosphate and the mitochondrial $NADH/NAD^+$ ratio.

In cardiac tissue the adenylate system is not a good indicator of the energy state of the mitochondrion, even when the concentrations of AMP and free cytosolic ADP are calculated from the adenylate kinase and creatine kinase equilibria. Only creatine phosphate and inorganic phosphate undergo significant changes, but evidence of the participation of the latter compounds in the regulation of the pyruvate dehydrogenase interconversions is lacking.

The potassium-induced arrest of the heart resulted in a decrease in pyruvate, a slight increase in acetyl-CoA, a large increase in the concentration of citrate and an increase in the mitochondrial $NADH/NAD^+$.

The results can be interpreted as showing that in the heart, the pyruvate dehydrogenase interconversions are mainly regulated by the pyruvate concentration and the mitochondrial redox state. Concentrations of all the regulators tested shifted

to directions which one would expect to result in a decrease in the amount of active pyruvate dehydrogenase, but the changes were quite small. Therefore, the energy-linked regulation of pyruvate dehydrogenase in intact tissue is possibly mediated by the equilibrium relations between the cellular redox state and the phosphorylation potential recently confirmed in cardiac tissue.

INTRODUCTION

The energy-producing pathways of carbohydrate metabolism are efficiently regulated to accommodate the ATP consumption of the cell. In a previous report from this laboratory, it was shown that in the isolated perfused heart, glycolysis and pyruvate oxidation are rapidly adjusted according to changes of the flux through the citric acid cycle [1]. Therefore, in the experiments to be reported here, attempts were made to estimate the role of regulation of the interconvertible enzyme pyruvate dehydrogenase in the adjustment of the rate of pyruvate oxidation.

Pyruvate dehydrogenase can exist in an active (dephosphorylated) or inactive (phosphorylated) form [2]. The relative amounts of these forms are regulated by pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphatase. The kinase inactivates pyruvate dehydrogenase by phosphorylation by $MgATP^{2-}$ and the activation of pyruvate dehydrogenase is achieved by hydrolysis of the phosphoenzyme by the pyruvate dehydrogenase phosphatase [3, 4]. The mechanism of regulation of this phosphorylation-dephosphorylation cycle is still a matter for discussion. The pyruvate dehydrogenase phosphatase requires free Mg^{2+} and Ca^{2+} for its activity [6, 5]. The K_m for Mg^{2+} is about 20 mM in phosphate buffer [7]. It is usually considered that the activity of pyruvate dehydrogenase is mostly regulated by the mitochondrial ATP/ADP ratio [8, 9]. This regulation by the adenylates may be mediated by the differing chelating properties of ATP, ADP and AMP and resulting changes in the free Mg^{2+} concentration, which is much lower than the K_m of pyruvate dehydrogenase phosphatase for Mg^{2+} [4]. ADP also acts as a weak inhibitor of the pyruvate dehydrogenase kinase. It has been shown in experiments with purified pyruvate dehydrogenase that the activity of pyruvate dehydrogenase is regulated by the acetyl-CoA/CoA and $NADH/NAD^+$ ratios [10–12], and the role of these regulators has also been shown in suspensions of liver mitochondria [13]. Pyruvate also affects the interconversions of pyruvate dehydrogenase by inhibiting the pyruvate dehydrogenase kinase [4]. In muscle mitochondria, citrate inhibits the dephosphorylation of inactive pyruvate dehydrogenase, and this effect is not mediated by chelation of Mg^{2+} [5]. It has also been suggested that α-oxoglutarate is implicated in the activation-deactivation cycle of pyruvate dehydrogenase, possibly by means of changes in the concentrations of the high energy compounds [14].

This multiplicity of the potential regulators makes any deductions about their mutual order of significance in the metabolic interlocks of intact cells and tissues difficult. Since the regulation of metabolism to conform with the cellular energy consumption may be considered as the most basic one, the approach of the present report was adopted. We wish to report here that when the energy state of the perfused heart is perturbed by changing its performance of mechanical work, the activation-deactivation cycle of pyruvate dehydrogenase is regulated in a coherent manner to

adjust its activity according to the terminal oxidations. The KCl-induced arrest of the heart was employed to abruptly achieve conditions of low ATP consumption and concomitant changes in the energy state of the myocardial cell.

EXPERIMENTAL

Reagents. Enzymes were obtained from Sigma Chemical Co, St Louis, Mo., U.S.A., and from Boehringer GmbH, Mannheim, Germany. [^{14}C]Pyruvate and NCS solution for CO_2 collection were obtained from the Radiochemical Centre, Amersham, England, and [^{3}H]glucose from NEN Chemicals GmbH, Dreieichenhain, Germany. Standard reagents were obtained from E. Merck AG, Darmstadt, Germany, nucleotides and coenzymes from Boehringer, GmbH, and dithiothreitol from Sigma Chemical Co.

Experimental animals and perfusion methods. Female Long-Evans rats from the department's own stocks were used. No fasting period preceded the experiments. The rats were anesthetized with intraperitoneal Nembutal and injected intravenously with 500 I.U. heparin 1 min before the excision of the heart. The hearts were perfused with Krebs-Ringer bicarbonate solution [15] containing 2.5 mM Ca^{2+} and 10 mM glucose in equilibrium with O_2/CO_2 (95 : 5, v/v), by Langendorff procedure [16] without recirculation at a hydrostatic pressure of 80 cm water. The oxygen concentration in the venous perfusate was monitored by a Radiometer E5046 electrode.

Glycolytic rate and liberation of $^{14}CO_2$ from [1-^{14}C]pyruvate in the heart perfusion. The rate of glycolysis was estimated by measuring the amount of tritiated water formed from [3-^{3}H]glucose, basically following the procedure of Safer and Williamson [17]. It was assumed that the 3-H of glucose equilibrates with water at the aldolase and triose phosphatase isomerase steps [18] with a complete loss of ^{3}H into the water. The apparatus described by Moss [19] was used for the collection of the tritiated water.

In order to measure the amount of ^{14}CO liberated from [1-^{14}C]pyruvate the effluent was collected under heptane at 1-min intervals. The effluent was acidified, shaken for 20 min in special vessels for the collection of the $^{14}CO_2$ released in NCS solution and the radioactivity was measured.

Heart extracts and protein determination. Samples were obtained from the heart using aluminium clamps cooled with liquid nitrogen [20]. Initial acid extraction from the frozen, pulverized sample was performed using 8 % (v/v) $HClO_4$ in 40 % (v/v) ethanol, precooled to −20 °C to ensure quenching in the frozen state [21]. Extraction was repeated with 6 % (v/v) $HClO_4$ and the filtrate neutralized to pH 6 with 3.75 M K_2CO_3 containing 0.5 M triethanolamine hydrochloride. The amount of protein in the $HClO_4$ precipitate was determined according to Lowry et al. [22] after dissolving the precipitate in NaOH solution.

Metabolites. The concentrations of metabolites in the samples were assayed by enzymatic methods, the formation or disappearance of NAD(P)H being measured in an Aminco DW-2 dual wavelength spectrophotometer. Citrate was measured with citrate lyase [23], α-oxoglutarate according to Narins and Passonneau [24], pyruvate according to Bücher et al. [25], glutamate according to Bernt and Bergmeyer [26], ammonia according to Kun and Kearney [27], CoA and acetyl CoA were measured with α-oxoglutarate dehydrogenase [28], which was prepared from pig heart

according to Sanadi et al. [29]. ADP and creatine were determined according Bernt et al. [30]. ATP was determined using hexokinase and glucose-6-phosphate dehydrogenase [31] and phosphocreatine in the same assay, by a subsequent addition of ADP and creatine kinase. Inorganic phosphate was measured with phosphorylase *a* essentially according to Gawehn [32].

Pyruvate dehydrogenase. The activity of pyruvate dehydrogenase was measured with radioactive [1-^{14}C]pyruvate. The rate of the liberation of CO_2 is proportional to the pyruvate dehydrogenase activity in cardiac tissue because of the low activity of pyruvate carboxylase [33, 34].

The samples (350–700 mg) for pyruvate dehydrogenase activity were obtained by the freeze-stop method and stored under liquid nitrogen until analyzed. The samples were homogenized with an Ultra-Turrax homogenizer in five volumes of ice-cold 10 mM potassium phosphate containing 1 mM EDTA, pH 7.4. An aliquot of the homogenate was immediately assayed for pyruvate dehydrogenase activity. To another aliquot, 0.1 M $MgCl_2$ was added to give a final concentration of 10 mM Mg^{2+}. The protein concentration was kept at about 25 mg/ml [35] and the mixture was incubated for 40 min at 25 °C to active pyruvate dehydrogenase.

The reaction was carried out in special vessels equipped with a side arm with an injection port for additions of trichloroacetic acid (see below), and with a device for the collection of CO_2 on a filter paper strip impregnated with NCS solution. The incubation temperature was 30 °C. The final reaction medium consisted of 69.6 mM potassium phosphate, pH 8.0, 0.17 mM EDTA, 1 mM dithiothreitol, 1.5 mM NAD^+, 1.4 mM $MgCl_2$, 4.3 mM [1-^{14}C]pyruvate (specific activity 12 000–15 000 dpm/μmol), 0.42 mM CoA-SH, 50 μg lactate dehydrogenase, 10 μg phosphotransacetylase and tissue homogenate in an amount equivalent to 3.0–4.0 mg protein. The total volume was 1.15 ml. The reaction mixture was first incubated at the stated temperature for 8 min, after which the reaction was started by addition of the sample in 150 μl. The reaction was stopped after 15 min with 0.5 ml of 10 % trichloroacetic acid. After an additional incubation for 10 min with continued shaking, the contents of the CO_2 collectors were rinsed into bottles containing 10 ml of a toluene-based scintillant.

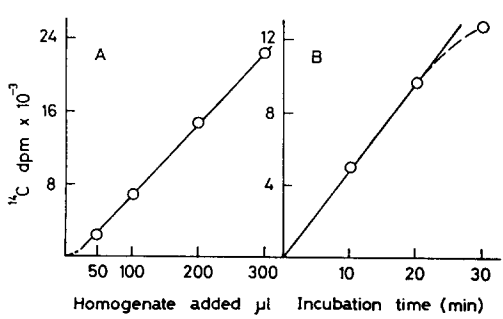

Fig. 1. Dependence of the pyruvate decarboxylation rate on the amount of homogenate and incubation time. The incubation mixture was the same as described under Experimental. (A) Effect of homogenate concentration. Incubation time 15 min. (B) Time course of the reaction. The amount of homogenate was 150 μl. Each point represents the mean of duplicate determinations.

The pyruvate decarboxylase activity was linearly proportional to the amount of tissue homogenate added (Fig. 1A) and the time course was linear for 20 min (Fig. 1B). The 15 min incubation time was therefore selected for the experiments. The unit of activity was taken as 1 μmol/min pyruvate decarboxylated.

RESULTS

In the present investigation, the mechanical work of the perfused heart was changed. The heart was arrested by raising the potassium concentration in the perfusion medium to 15 mM. It stopped within 2 min, and a rapid inhibition of oxygen consumption followed.

Glycolytic flux and oxygen consumption. In an open perfusion, the difference between the glucose concentrations in the arterial and venous perfusate is too small to be accurately measured. The rate of glycolysis was therefore estimated by measuring the amount of tritiated water formed from [3-^{3}H]glucose as explained under Experimental. As can be seen in Table I, the regulation of glycolysis is very efficient and rapid. 10 min after the arresting of the heart, the flux of glycolysis was inhibited by 70 %.

From the data on glycolytic flux and lactate plus pyruvate output (Table I), the rate of aerobic glucose oxidation can be calculated. The latter is inhibited by 91 % when the oxygen consumption is inhibited by 58 % only. This may indicate that the heart switches to the oxidation of fatty acids when the mechanical work diminishes. The data also indicates that almost all the oxygen consumption in the beating heart under these experimental conditions can be accounted for by the oxidation of glucose, and in the arrested heart the glucose-linked oxygen consumption is only about 30 %.

TABLE I

OXYGEN CONSUMPTION, GLYCOLYTIC FLUX, LACTATE AND PYRUVATE OUTPUT AND GLUCOSE OXIDATION IN ISOLATED PERFUSED POTASSIUM-ARRESTED RAT HEART

The values are the mean ±S.E. of five separate experiments. In each experiment all the parameters presented were measured from the same heart. Lactate and pyruvate were measured from the effluent perfusion fluid pooled for 1-min intervals.

Time after arrest (min)	O_2 consumption (μmol/min per g dry weight)	Glycolysis (μmol/min per g dry weight)	Lactate + pyruvate output (μmol/min per g dry weight)	Glucose oxidation (μmol/min per g dry weight)	Glucose-dependent oxygen consumption (%)
0	23.7±0.73	7.58±0.96	7.30	3.93	100
2	22.9	6.23	4.59	3.94	100
3	14.8	4.85	3.90	2.90	100
4	12.8	3.76	3.12	2.20	100
5	11.0	3.14	3.27	1.51	82
6	10.9	2.70	3.93	0.74	41
7	10.5	2.61	3.93	0.65	37
8	8.9	2.45	4.13	0.39	26
9	9.5	2.24	3.74	0.37	23

$^{14}CO_2$ production from [1-^{14}C]*pyruvate.* In order to study the flux through pyruvate dehydrogenase, the hearts were perfused with 10 mM glucose plus 0.2 mM [1-^{14}C] pyruvate. Low concentrations of pyruvate were used to prevent the inhibition of pyruvate dehydrogenase kinase by external pyruvate. On the other hand, the exogenous pyruvate concentration must be high enough to prevent excessive dilution effects due to endogenous production of pyruvate. The $^{14}CO_2$ liberation is proportional to the flux through the pyruvate dehydrogenase step, because pyruvate carboxylase activity is low in the heart tissue. The oxidation of external pyruvate decreased rapidly after the heart arrest, from about 0.650 to 0.168 μmol/min per g dry weight in 4 min, and to 0.120 μmol/min per g dry weight in 10 min (Fig. 2).

The results show that the flux through pyruvate dehydrogenase is very effectively regulated by the energy state of the tissue. It is also noticeable that the pyruvate oxidation is inhibited to a greater extent (82 %) than the glycolytic flux (73 %) or oxygen consumption (58 %); this data is in accord with the data on aerobic glucose oxidation presented in Table I. However, the values of [^{14}C]pyruvate oxidation are smaller than those calculated from the glycolytic flux and the lactate-plus-pyruvate output. Glycogenesis did not occur under these experimental conditions (not shown), so that this discrepancy is obviously due to dilution of the [^{14}C]pyruvate by pyruvate formed from exogenous glucose.

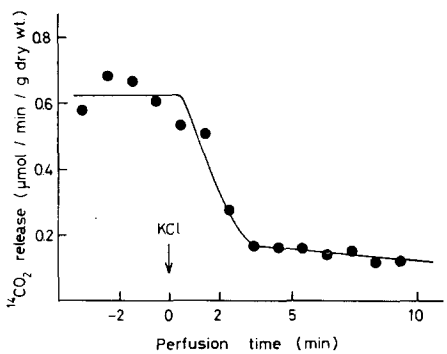

Fig. 2. $^{14}CO_2$ release in an isolated perfused rat heart perfused with Krebs-Ringer bicarbonate containing 0.2 mM [1-^{14}C]pyruvate and 10 mM glucose. The pre-perfusion time was 20 min and at time point zero, the KCl concentration in the perfusion fluid was raised to 15 mM. The specific activity of the pyruvate was 23 600 dpm/μmol.

Pyruvate dehydrogenase activity. In intact tissue, changes in the flux through the pyruvate dehydrogenase site could be due either to changes in the amount of the active form of the enzyme or to effects of substrate availability or modulation of the enzyme activity by mechanisms not involving enzyme interconversions. To test these alternatives, the portion of pyruvate dehydrogenase existing in the active form (pyruvate dehydrogenase a) was measured. The pyruvate dehydrogenase a content was found to be 4.89±0.54 munits/mg protein in the beating heart after 20 min perfusion with medium supplemented with 10 mM glucose. The maximum activity when all pyruvate dehydrogenase was converted to pyruvate dehydrogenase a was 23 munits/mg protein. A rapid decrease in the pyruvate dehydrogenase a content occurred after the KCl-

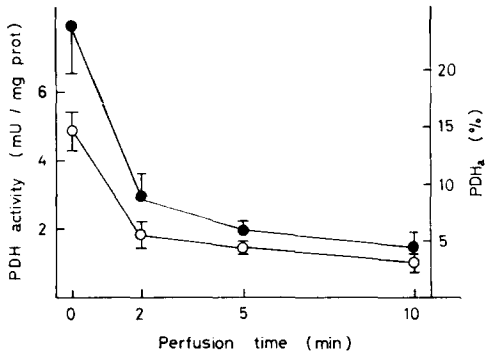

Fig. 3. Effects of the cardiac arrest on the pyruvate dehydrogenase activity in the isolated perfused rat hearts. The heart was first perfused with Krebs-Ringer bicarbonate (containing 10 mM glucose) for 20 min and the perfusion with the same fluid containing 15 mM KCl was begun at the time marked 0 min (Mean ±S.E., 3–5 separate experiments). ○–○, activity in munits/mg protein; ●–●, activity in percent of the fully activated enzyme.

induced arrest of the heart, already evident 2 min after stopping the heart (Fig. 3). When these results (78 % inhibition at 10 min) are compared with those of pyruvate oxidation, one finds that the percentage inhibition of pyruvate oxidation and also the time course of the inhibition is almost the same as the percentage decrease in the amount of pyruvate dehydrogenase a. The results suggest that the amount of pyruvate dehydrogenase a might be the main regulator of pyruvate oxidation in the perfused heart.

Contents of pyruvate, acetyl-CoA and CoA-SH. After the cardiac arrest, the content of pyruvate in the tissue decreased gradually from 0.706±0.071 to 0.534± 0.071 nmol/mg protein in 10 min. Under similar experimental conditions, the acetyl-CoA content increased from 0.053±0.003 to 0.170±0.031 nmol/mg protein. There were no marked changes in the tissue free CoA-SH content (Table II).

It could be expected that a diminishing flux through the citric acid cycle would

TABLE II

PYRUVATE, CoA-SH, ACETYL-CoA AND α-OXOGLUTARATE CONCENTRATIONS AND MITOCHONDRIAL NADH/NAD$^+$ RATIO IN ISOLATED PERFUSED POTASSIUM-ARRESTED RAT HEART

The values are the mean ±S.E. of 4–8 separate experiments.

Time after arrest (min)	Pyruvate (nmol/mg protein)	CoA-SH (nmol/mg protein)	Acetyl-CoA (nmol/mg protein)	α-Oxoglutarate (nmol/mg protein)	Mitochondrial NADH/NAD$^+$ ratio*
0	0.706±0.71	0.700±0.047	0.053±0.003	0.738±0.056	0.722±0.060
2	0.680±0.031	0.730±0.047	0.086±0.011	0.632±0.047	0.921±0.064
5	0.665±0.062	0.680±0.036	0.113±0.017	0.667±0.075	1.050±0.163
10	0.534±0.071	0.700±0.038	0.170±0.031	0.726±0.032	1.057±0.077

* $\text{NADH/NAD}^+ = \dfrac{3.87 \times [\text{glutamate}]}{10^3 \times [\alpha\text{-oxoglutarate}][\text{NH}_3]}$.

result in an increase in the concentration of acetyl-CoA. However, the changes in the acetyl-CoA concentration are quite small, which indicates that the acetyl-CoA producing pathways, pyruvate oxidation and β-oxidation of the fatty acids are inhibited simultaneously. The decrease in the pyruvate concentration is probably due to both a decrease in the glycolytic flux and a small change in the cellular redox state (see below).

The mitochondrial NADH/NAD$^+$ ratio and the contents of citrate and α-oxoglutarate in the heart. To measure the mitochondrial free NADH/NAD$^+$ ratio in the heart is a difficult task, because either strictly mitochondrial NAD-linked dehydrogenases have low activity, or the corresponding redox substrate pairs are not in equilibrium in the heart tissue. β-Hydroxybutyrate dehydrogenase is not suitable because acetoacetic acid is a reductant in heart tissue [36]. Glutamate dehydrogenase is the only choice, but its activity is also relatively low in the heart mitochondria [17].

As calculated from the glutamate dehydrogenase equilibrium, the mitochondrial NADH/NAD$^+$ ratio increased rapidly (Table II), reaching a new steady-state value within 2 min. This is in accord with the time course of the redox changes as detected by surface fluorimetry of the heart [1].

Citrate has been reported to modulate the activity of pyruvate dehydrogenase with a mechanism not involving complexing of Mg^{2+} [5]. We measured the time course of the changes of myocardial citrate, which increased in 2 min from the initial 0.98 ± 0.12 to 1.85 ± 0.43 nmol/mg protein and reached a maximum of 2.28 ± 0.29 nmol/mg protein in 10 min after the KCl-induced arrest of the heart (Fig. 4).

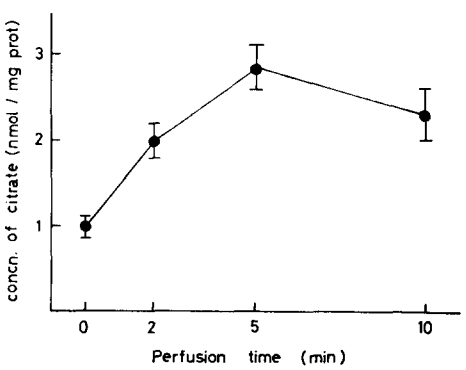

Fig. 4. Changes in the citrate content of the isolated perfused rat heart after the cardiac arrest with 15 mM KCl. The experimental conditions were as in Fig. 3. (Mean ±S.E., 3–12 separate experiments).

The α-oxoglutarate content of the myocardial tissue showed quite small changes after the cardiac arrest. After an initial decrease at 2 min, the content tended to increase (Table II).

Concentrations of the adenylates and inorganic phosphate in the cardiac tissue. Pyruvate dehydrogenase is known to be regulated by the energy state of the mitochondria. It has been suggested that the effects of changing the ATP/ADP ratio on the pyruvate dehydrogenase phosphatase are mediated through the differential chelating properties of the adenine nucleotides for free Mg^{2+} [7].

TABLE III

CONCENTRATIONS OF THE ADENINE NUCLEOTIDES, INORGANIC PHOSPHATE AND THE CREATINE PHOSPHATE/CREATINE RATIO IN BEATING AND POTASSIUM-ARRESTED HEARTS

The results are mean ±S.E. of five separate experiments and expressed as μmol/g wet weight.

	ATP		ADP_f[a]		AMP[b]		P_i	Phosphocreatine/creatine	
	Beating heart	Potassium-arrested heart	Beating heart	Potassium-arrested heart	Beating heart	Potassium-arrested heart	Potassium-arrested heart	Beating heart	Potassium-arrested heart
0							2.58±0.12		
2							2.22±0.30		
5							1.82±0.11		
10	4.26±0.31	4.21±0.42	0.174±0.021	0.143±0.018	0.0029±0.005	0.0025±0.005	2.11±0.39	1.65±0.115	1.89±0.101

[a] ADP_f: free cytosolic ADP, calculated from the phosphocreatine (CP)/creatine (C) ratio and total tissue ATP concentration, assuming that the creatine kinase reaction is in equilibrium.
$K_{eq} = ([ATP^{4-}][C])/([H^+][ADP^{3-}][CP]) = 1.51 \cdot 10^8\ M^{-1}$.

[b] AMP: calculated from the ATP concentration and the phosphocreatine/creatine ratio, the creatine kinase and adenylate kinase reactions taken to be in equilibrium [37].

To test these possibilities in the perfused heart, the concentrations of the adenylates and inorganic phosphate were determined. In muscle tissue, the determination of the concentration of free ADP comes up against some difficulties because of the high concentration of cytosolic binding sites for this nucleotide. Therefore, the concentrations of creatine phosphate and creatine were also determined and the cytosolic free ATP/ADP ratio was calculated from the creatine kinase equilibrium [37].

As expected, the ATP/ADP ratio showed quite small changes, which are far too small to be effective in the adenylate-linked regulation of pyruvate dehydrogenase (Table III). The concentration of inorganic phosphate showed the largest percentage changes, equivalent to the changes in creatine phosphate.

It could be expected that the mitochondrial ATP/ADP ratio would show proportional changes similar to the cytosolic ATP/ADP ratio, on the basis of the demonstrated equilibrium relations between the extramitochondrial phosphorylation potential and the free energy relations in the mitochondrial respiratory chain. This would mean that the mitochondrial changes in the ATP/ADP ratio are also small. The concentration of inorganic phosphate is therefore the main determinant of the mitochondrial energy state in cardiac tissue. However, studies with isolated liver mitochondria [38] have shown that inorganic phosphate does not have much regulatory effect on pyruvate dehydrogenase.

AMP occurs in the lowest concentrations of the adenylates and, as it also presents the breakdown product of other adenylates, its determination is subject to large artifacts. The concentration of AMP was therefore calculated from the cytosolic free ADP concentration and the adenylate kinase reaction, the latter being assumed to be in equilibrium in heart tissue. The data which is presented in Table III, shows that the concentration of AMP changes only by 23 % during the potassium-induced cardiac arrest. This change can be considered quite small when compared with the drastic changes in the glycolytic flux.

The total ADP concentration in the heart tissue was 0.668 and 0.623 μmol/g wet weight in the beating and arrested heart, respectively. Comparison of this data with that of Table III gives the important parameter of the concentration of the high affinity ADP binding sites, which is 0.490 μmol/g wet weight in the cardiac tissue. This value is probably more accurate than the previously published estimations based on differential extraction of ADP from muscle tissue [39].

DISCUSSION

In the fuel economy of biological systems, the regulatory mechanisms of the energy-yielding metabolic pathways have a fundamental role. The phenomenon of "respiratory control" [40] at the level of the terminal oxidations must be supplemented with efficient mutual and sequential regulation between the anaerobic and oxygen-consuming ATP-producing pathways. This mutual regulation is exemplified by the Pasteur and Crabtree effects [41, 42]. Moreover, this mutual regulation must be effective for a simultaneous feedback regulation to inhibit needless accumulation of intermediary metabolites throughout the metabolic sequence.

Recently, we took advantage of the rapid changes in the ATP consumption of the isolated perfused heart to study the phenomenon of respiratory control in intact

tissues [1]. The results also indicated that the feed-back regulation of the fuel breakdown to metabolites of the intermediary energy metabolism must have an extraordinary capacity. The present results show that the glycolytic flux measured with exogenous glucose is very effectively regulated. Moreover, the glycolytic flux decreases more than the oxygen consumption during diminution of ATP consumption of the muscle. This actually means that a switch-over from carbohydrate to lipid substrates occurs in the heart muscle when the mechanical work decreases.

Almost the same percentage decrease in the pyruvate dehydrogenase activity was observed as in the glycolytic flux. Under these experimental conditions (open perfusion), pyruvate oxidation was not necessarily limiting for the glucose utilization because of the great losses of pyruvate and lactate in the perfusate. However, the results still show that the activity of pyruvate dehydrogenase is regulated in pace with the glycolysis and oxygen consumption to effectively keep a relative constancy of the metabolite concentrations.

Several possibilities exist for the regulation of pyruvate dehydrogenase. As shown in the present paper, no single mechanism seems to be predominant in the cardiac tissue. Some conclusions concerning their roles can, however, be drawn on the basis of the temporal pattern of the concentration changes of the potential regulators, compared with the time courses of the changes the pyruvate dehydrogenase activity.

In the myocardium, the regulation by the mitochondrial energy state is also complicated by the high phosphagen content of the tissue. The cytosolic ATP/ADP ratio shows little change during the cardiac arrest, as can be expected on the basis of the creatine phosphate content of the myocardium. The fractional change in the mitochondrial ATP/ADP ratio is possibly the same as in the cytosol. Therefore, it is highly unlikely that the energy state, as reflected solely by the adenylate system, has a major role in the regulation of pyruvate dehydrogenase in cardiac tissue. The energy state defined as the phosphorylation potential does change, but the causal relation between the latter and pyruvate dehydrogenase activity remains to be validated. There is no in vitro evidence of the involvement of inorganic phosphate in the regulation of pyruvate dehydrogenase [38]. The regulation by the adenylates evidently needs some kind of an amplification system to be effective. It has been suggested that this amplification for phosphofructokinase is provided by the adenylate kinase in heart tissue. However, the evidence is based on rather drastic changes of the metabolism such as are effected by anaerobiosis, respiratory inhibitors and uncouplers of oxidative phosphorylation.

It has already been suggested that the pyruvate dehydrogenase kinase is more sensitive to pyruvate in the heart tissue than in other tissues (K_i of pyruvate = 0.08–0.3 mM) [4]. The pyruvate concentration decreased from 0.092 mM in the beating hearts to 0.069 mM in the hearts arrested for 10 min. The increase in the acetyl-CoA concentration is rather small and the change in the acetyl-CoA accumulation slow compared with the rapid inactivation of pyruvate dehydrogenase. Thus, it is unlikely that acetyl-CoA has a significant role in the inactivation of pyruvate dehydrogenase under these experimental conditions.

The increase in the $NADH/NAD^+$ ratio is a change known to result in pyruvate dehydrogenase inactivation in mitochondria [10–12]. As estimated from the glutamate dehydrogenase equilibrium, the change in the NADH concentration is quite small. However, marked changes in the NAD(P)H fluorescence in the myocardium can be

observed by organ fluorimetry of the perfused heart during KCl-induced arrest [1]. This might indicate that the change in the NADH/NAD^+ ratio calculated from the glutamate dehydrogenase equilibrium is only a minimum value, and that the change in fact is large enough to affect the pyruvate dehydrogenase interconversions. It has also recently been pointed out by Opie and Owen [43] that the interpretation of calculations of the mitochondrial NADH/NAD^+ ratio in heart tissue must be tempered by some reservations. We studied the effects of acetoacetate on the cellular redox state by organ fluorimetry and found a reduction of NAD(P)H by acetoacetate (Hassinen, I. E., unpublished). This is direct evidence that the β-hydroxybutyrate/acetoacetate ratio is not a redox indicator in cardiac tissue.

The concentration of citrate in the tissue increased about 2-fold. This change has also the additional effect of converting pyruvate dehydrogenase to the inactive form [5]. The citrate/isocitrate ratio reflecting the aconitase equilibrium also increases. The aconitase equilibrium is known to be affected by the Mg^{2+} concentration [44]. However, the large increase in the citrate concentration makes any deductions concerning the free Mg^{2+} concentration on the basis of the aconitase equilibrium unreliable, because of the greatly increased Mg^{2+} binding power of the citrate under these conditions. This binding leads to the formation of magnesium citrate. As pointed out by Veloso et al. [44], the citrate/isocitrate ratios usually observed in perfused hearts using exogenous glucose as the substrate are not permissible values, as judged on the basis of in vitro equilibrium data. On the other hand, a mechanism of pyruvate dehydrogenase inactivation by citrate not involving complexing of magnesium has been suggested [5].

α-Oxoglutarate has also been implicated in the regulation of pyruvate dehydrogenase in the liver [14]. The mechanism proposed is, however, highly speculative. The observed changes in the α-oxoglutarate concentration in the heart are probably too small to have any significance in the regulation of pyruvate dehydrogenase in the heart.

The percentage changes in the pyruvate oxidation in the perfused heart and the percentage changes in the amount of active pyruvate dehydrogenase are almost identical. This suggests that pyruvate dehydrogenase is rate limiting for the pyruvate oxidation, and is also a proof of the observation that the activity determinations of pyruvate dehydrogenase in vitro very closely reflect the activity of pyruvate dehydrogenase in situ.

This experimental model demonstrates a co-ordinate control of a metabolic pathway, in which control points at three levels operate in pace to stabilize effectively the intermediary metabolite concentrations. This regulation still shows the co-ordinate behaviour in spite of the washing-out of the metabolites in the open perfusion system. The somewhat lower pyruvate dehydrogenase a content in the heart in open perfusion, without added pyruvate compared with the cardiac pyruvate dehydrogenase a content in vivo, is probably due to the washing-out of pyruvate from the heart. The regulation also seems to operate with extraordinarily small changes in the effector concentrations, which is possibly evidence of an amplification system. The net effects of the kinase and phosphatase enzymes on pyruvate dehydrogenase actually provide an amplification system of the pyruvate dehydrogenase regulation with the phosphorylation-dephosphorylation cycle. It also seems plausible to assume that the same amplification systems do not operate for both glycolysis and pyruvate dehydrogenase. The adenylate

kinase reaction is usually considered to be the most important amplifier of the energy state effect of the cell on phosphofructokinase [45]. The 23 % change in the AMP concentration may indeed be sufficient to regulate the phosphofructokinase step. However, the role of the latter in the regulation of pyruvate dehydrogenase remains to be established.

The heart mitochondrial membrane is rather impermeable to citrate, but LaNoue et al. [46] have shown that if the mitochondrial concentration of citrate increases to a certain value, citrate flows out from mitochondria. Arresting of the heart causes a marked increase in the citrate concentration, which under these conditions are indicative of diminished flux through the citric acid cycle. This increase could be high enough to cause the feed-back inhibition of phosphofructokinase in the cytoplasmic space.

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