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COMPARTMENTATION OF CITRATE IN RELATION TO THE REGULATION OF GLYCOLYSIS AND THE MITOCHONDRIAL TRANSMEMBRANE PROTON ELECTROCHEMICAL POTENTIAL GRADIENT IN ISOLATED PERFUSED RAT HEART

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Subcellular fractionation of tissue in nonaqueous media was employed to study metabolite compartmentation in isolated perfused rat hearts. The mitochondrial and cytosolic concentrations of citrate and 2-oxoglutarate, total concentrations of the glycolytic intermediates and rate of glycolysis were measured in connection with changes in the rate of cellular respiration upon modulation of the ATP consumption by changes of the mechanical work load of the heart. The concentrations of citrate and 2-oxoglutarate in the mitochondria were 16- and 14-fold, respectively, greater than those in the cytosol of beating hearts. The cytosolic citrate concentration was low compared with concentrations which have been employed in demonstrations of the citrate inhibition of glycolysis. In spite of the low activities reported for the tricarboxylate carrier in heart mitochondria, the cytosolic citrate concentration reacted to perturbations of the mitochondrial citrate concentration, and inhibition of glycolysis at the phosphofructokinase step could be observed concomitantly with an increase in the cytosolic citrate concentration. The ΔpH across the inner mitochondrial membrane calculated from the 2-oxoglutarate concentration gradient and the mitochondrial membrane potential calculated from the adenylate distribution gave an electrochemical potential difference of protons compatible with chemiosmotic coupling in the intact myocardium.

Introduction

The glucose-sparing effect of free fatty acis in muscle tissue has been accepted as a general principle involved in the adaptation to starvation and fat feeding [1]. This concept is based on the observation of inhibition of glucose oxidation by elevated extracellular free fatty acid concentration in isolated perfused hearts [2]. The mechanism of this inhibition is not known with any certainty. The first explanation was that citrate is the key regulator of phosphofructokinase in muscle and, therefore, the accumulation of citrate during the oxidation of ketone bodies or fatty acids is the reason for the decrease in glucose oxidation under these conditions [3]. This tentative regulatory mechanism reaches a dilemma when applied to heart muscle, viz., the compartmentation of citrate, which is reinforced by the very low activity of the tricarboxylate translocator [4–6], implying that the citrate concentration increase is confined to mitochondria, whereas phosphofructokinase is cytosolic.

The relative importance of the different effectors of phosphofructokinase under conditions prevailing in vivo is not known. Glycolytic flux has been demonstrated as fluctuating within a large range in heart muscle although the concentrations of the adenylates are kept within narrow limits

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[7,8], and an accumulation of citrate coincides with the inhibition of glycolysis. Recent reports on the regulation of phosphofructokinase by covalent enzyme interconversions [9,10], which yield an effective amplification mechanism of control, and the newly discovered regulator fructose 2,5-diphosphate [11], add a new dimension to the regulation of phosphofructokinase.

To advance knowledge of the relative importance of the various possible regulators of phosphofructokinase in myocardium, we set out to study the compartmentation of citrate during inhibition of glycolysis in isolated, perfused rat hearts on a change in the rate of ATP consumption due to changes in the mechanical work load of the heart. The results demonstrate that the cytosolic citrate concentration is very low compared with the concentrations used in demonstrating citrate inhibition of phosphofructokinase and the cytosolic concentration is a small fraction of the mitochondrial citrate concentration. The cytosolic citrate concentration, however, increases with a decrease in the cellular ATP consumption.

Experimental Procedure

Reagents

The enzymes, nucleotides, L-malic acid, citric acid and glyceraldehyde 3-phosphate were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G. and Sigma Chemical Co., St. Louis, MO, U.S.A. D-[3-^{3}H]Glucose was purchased rom the Radiochemical Centre, Amersham, Bucks, U.K. Other reagents were purchased from E. Merck AG, Darmstadt, F.R.G.

Animals and perfusion methods

Female Sprague-Dawley rats weighing 200–230 g from this Department's own stock were used. No fasting period preceded the experiments. Animals were anesthetized with intraperitoneal pentobarbital (60 mg/kg, Mebumat^R, Orion Pharmaceutical Co., Finland). 500 IU of heparin were injected intravenously 30 s before excision of the heart. Isolated hearts were perfused without recirculation by the Lagendorff procedure with Krebs-Ringer bicarbonate solution [12] containing 2.5 mM $CaCl_2$ and 10 mM glucose and equilibrated with O_2/CO_2 (19:1). The perfusion pressure was 7.85 kPa (80 cm water). After a preperfusion of 15 min the hearts were perfused for another 15 min with the same solution (beating hearts) or with a medium containing 16 mM KCl, the Na^+ concentration being accordingly reduced to keep osmolarity constant (arrested hearts).

Oxygen consumption and glycolytic rate

Oxygen consumption was calculated from the oxygen concentration difference between the influent and effluent perfusion solution measured by a Clark-type electrode (Radiometer E5046) and from the flow of the perfusate collected in 1-min fractions. The rate of glycolysis was estimated from the amount of ^{3}H liberated from D-[3-^{3}H]glucose (spec. act. 12000 dpm/μmol) into water [13]. ^3H_2O was distilled with an apparatus described by Moss [14] and radioactivity was counted in Lumagel.

Citrate compartmentation

The heart was quickly frozen by using aluminum clamps cooled with liquid nitrogen [15] and powdered and freeze-dried at −55°C for 72 h. The dry tissue powder was homogenized by means of sonication (MSE 60 W 19 kHz sonicator, Measuring & Scientific Equipment Ltd, London, U.K.) into heptane/carbon tetrachloride (50:50, v/v) at −25°C. The total sonication time was 15 min in 15-s bursts at maximum output separated by 30-s intervals for cooling.

The samples were prepared further principally as described by Elbers et al. [16] with minor modifications as described by Kauppinen et al. [8]. After centrifugation (27000 × g for 2 h, Spinco SW-41 rotor) in a discontinuous gradient of heptane in carbon tetrachloride (specific gravity 1.28–1.33) fractions were collected and divided into two portions which were evaporated to dryness. The samples for the enzyme assays were dispersed into 20 mM potassium phosphate buffer, pH 7.2, with a Braun Microdismembranator (B. braun, Melsungen, F.R.G.). Citrate syntase was assayed as described by Shepherd and Garland [17] and phosphoglycerate kinase according to the method of Bücher [18]. Protein was determined by the method of Lowry et al. [19]. from the other portion of the fractions acid-soluble metabolites were extracted with ice-cold perchloric acid (8%, v/v).

Citrate and 2-oxoglutarate in neutralized perchloric acid extracts of the fractions were determined by the methods of Gruber and Moellering [20] and Narins and Passonneau [21], respectively, which were modified by measuring NAD^+ formation by the alkaline fluorescence-enhancement method [22,23]. The final assay mixture consisted of 5.5 mM triethanolamine, pH 7.2, 1 mM $MgSO_4$, 0.5 mM EDTA, 85 μM ascorbate, 15 μM NADH, 0.1 mU citrate lyase/ml, 5 μg malate dehydrogenase/ml, total volume 120 μl. The concentration of citrate was below 20 μM. Prior to use the NADH stock solution (in carbonate buffer, pH 10) was heated for 15 min at 60°C to destroy contaminating NAD^+ [24]. The reaction time in the assay was 15 min, after which NADH was destroyed by lowering the pH to 1.0 with HCl. After 5 min, 1 ml of 6 M NaOH was added and the mixture was incubated for 1 h in darkness. Fluorescence excited at 365 nm was measured in an Aminco filter fluorometer (American Instrument Co., Silver Springs, MD, U.S.A.).

Glycolytic intermediates

The intermediates of glycolysis were determined in separate experiments on isolated perfused hearts of which neutralized perchloric acid extracts of freeze-clamped tissue were made without the freeze-drying procedure. Glucose 6-phosphate and fructose 6-phosphate were determined as described by Hohorst [25] and glucose 1-phosphate by the method of Bergmeyer and Michal [26]; fructose 1,6-diphosphate, dihydroxyacetophosphate and glyceraldehyde 3-phosphate according to Bücher and Hohorst [27], phospho*enol*pyruvate and pyruvate according to Czok and Lamprecht [28] and lactate as described by Hohorst [29]. Analyses were begun immediately after obtaining the neutralized extract.

Results

Compartmentation of citrate and 2-oxoglutarate

Myocardial citrate was found to be located mainly in mitochondria, where its concentration was 16-times higher than that in the cytosol (Table I). The tissue citrate concentration was subject to modulation by the cellular energy state, the concentration being more than 2-fold greater in hearts arrested by perfusion with the high-K^+

TABLE I

OXYGEN CONSUMPTION, CITRATE COMPARTMENTATION, RATE OF GLYCOLYSIS AND CONCENTRATIONS OF GLYCOLYTIC INTERMEDIATES IN ISOLATED PERFUSED RAT HEARTS

Experimental conditions as explained in Experimental Procedure. The values are means ± S.E. for the number of experiments given in parentheses.

	Beating heart			K^+-arrested heart		
Values (μmol·min^{-1}·g^{-1} dry wt.)						
Oxygen consumption	23.2	± 1.2	(5)	7.95	± 1.15 [c]	(6)
Values (mM)						
Citrate, mitochondrial	1.25	± 0.24	(6)	3.02	± 0.36 [c]	(4)
cytosolic	0.080	± 0.007	(6)	0.22	± 0.06 [a]	(4)
2-Oxoglutarate, mitochondrial	1.57	± 0.03		2.33	± 0.31	
cytosolic	0.14	± 0.07		0.23	± 0.06	
Values (nmol·g^{-1} dry wt.)						
Glucose 1-phosphate	53.4	± 4.5	(10)	75.3	± 4.5 [c]	(8)
Glucose 6-phosphate	416	± 50	(9)	573	± 65	(8)
Fructose 6-phosphate	98.3	± 7.8	(10)	148	± 19 [a]	(8)
Fructose 1,6-diphosphate	372	± 30	(10)	272	± 34 [a]	(8)
Dihydroxyacetone phosphate	158	± 18	(9)	105	± 6 [b]	(8)
Glyceraldehyde 3-phosphate	27.9	± 3.2	(10)	23.4	± 2.2	(8)
Phospho*enol*pyruvate	26.2	± 4.6	(10)	21.9	± 4.0	(8)
Pyruvate	130	± 13	(10)	215	± 5 [c]	(8)
Lactate	7208	± 1136	(9)	3491	± 811 [a]	(7)

P (vs. beating heart; student's *t*-test): [a] $P<0.05$, [b] $P<0.01$, [c] $P<0.005$.

medium. Also, under the latter conditions, citrate was mainly confined to mitochondria. However, the percentage increase in the citrate concentration was almost the same in the mitochondria and in the cytosol.

Also, the concentration of 2-oxoglutarate was higher in the mitochondria than in the cytosol.

Oxygen consumption

The transition from activity to rest, induced by the high-K^+ medium, caused a 66% inhibition in oxygen consumption (Table I), corroborating previous reports of experiments performed under similar conditions [30].

Glycolytic rate and concentrations of the intermediates of glycolysis

Cessation of the mechanical activity caused a 77% inhibition of the unidirectional glycolytic utilization of external glucose (Table I). A crossover plot of the concentration changes of the glycolytic intermediates (Fig. 1) demonstrates inhibition of glycolysis at the phosphofructokinase step. The lactate/pyruvate ratio was higher in the beating than the arrested hearts.

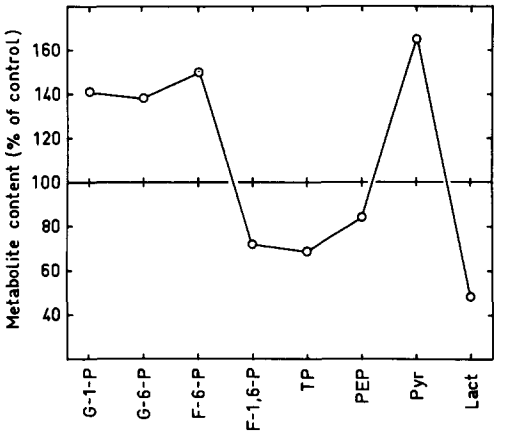

Fig. 1. Crossover plot of the concentrations of glycolytic intermediates during KCl-induced respiratory inhibition in isolated perfused rat hearts. The metabolic concentrations in KCl-arrested, isolated perfused rat hearts are expressed as percentage of the corresponding value in beating, isolated perfused hearts. G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose 6-phosphate; F-1,6-P, fructose 1,6-diphosphate; TP, triose phosphates (dihydroxyacetone phosphate + glyceraldehyde 3-phosphate); PEP, phospho*enol*pyruvate; Pyr, pyruvate; Lact, lactate.

Discussion

The participation of citrate in the regulation of glycolysis is still a somewhat controversial issue. The recent thorough study by Wu and Davis [31], however, indicates that rather low citrate concentrations can inhibit glycolysis, provided that the P_i concentration is low. Taking into account the cytosolic P_i concentrations [8] in perfused hearts, the cytosolic citrate concentrations reported above and the results of Wu and Davis [31], the metabolic transition used in the present study should lead to a 40% inhibition of glycolysis. This percentage is low compared with the 77% decrease in the glycolytic flux actually measured. This indicates that citrate is not the only regulator of glycolysis under these conditions.

The crossover plot depicted in Fig. 1 demonstrates a crossover point between fructose 6-phosphate and fructose 1,6-diphosphate, indicating control of glycolysis at the phosphofructokinase step. The other apparent crossover between pyruvate and lactate is more difficult to explain. Fig. 1 which represents a work-to-rest transition is almost a mirror image of the crossover plot obtained by Kobayashi and Neely [32] who studied a low work-to-high work transition. In the latter case [32], the increase in the lactate/pyruvate ratio is due to limitation of the reoxidation of glycolytic NADH, but the cause of the apparently similar phenomenon in the experimental model used in the present study needs further documentation.

The mitochondrial/cytosolic concentration gradient of citrate in the myocardium is higher than in perfused rat liver where it is only 5–9 [32]. The present study demonstrates a concentration gradient of 13–16 which, however, is comparable to the ratio found with the digitonin fractionation method in isolated hepatocytes [34]. Since the activity of the tricarboxylate translocase may be low [6], one cannot say with certainty whether an equilibrium of the citrate concentrations exists across the inner mitochondrial membrane. If an equilibrium exists, some interesting interconnections between membrane phenomena and the cellular energy state can be calculated. Given that the distribution of citrate is determined by the distribution of H^+ according to the notion that the membrane is permeable to the undissociated acid

[35], the pH difference across the membrane is $\Delta pH = 1/3 \log([C_m]/[C_c])$ where $[C_m]$ and $[C_c]$ are the mitochondrial and cytosolic concentrations of the anion. The ΔpH becomes 0.40 (alkaline inside) in the beating heart and 0.38 (alkaline inside) in the arrested heart. When the membrane potential is calculated from the adenylate distribution assuming that the ATP-ADP exchange is mainly electrogenic [36], using values published previously for similar conditions and methods [8], the mitochondrial membrane potential $\Delta\psi$ is 125 mV (negative inside) in the beating heart and 168 mV in the arrested heart. The electrochemical potential of H^+ would then be equal to $\Delta\tilde{\mu}_{H^+} = F\Delta\psi + 2.303RT\Delta pH = 14.4$ kJ · mol^{-1} in the beating heart and 18.4 kJ · mol^{-1} in the arrested heart. The cytosolic ΔG of ATP hydrolysis under the same conditions has been reported to be -58.8 kJ · mol^{-1} in the beating heart and -61.4 kJ · mol^{-1} in the arrested heart [8]. If the ATP synthesis is driven by an H^+-pumping ATPase [37], the observed H^+ electrochemical potentials and phosphorylation potentials give H^+/ATP ratios between 3.3 and 4.0 for the ATPase, ratios which indeed are near the range reported for intact isolated mitochondria [38,39].

Although the activity of the tricarboxylate translocase is low in heart mitochondria, the activity reported by Cheema-Dhadli et al. [6] yields a calculated myocardial total activity of 29 nmol · min^{-1} · g^{-1} wet weight, which is in excess of the rate needed (5 nmol · min^{-1} · g^{-1} wet weight) for the citrate concentration changes observed in the present study.

Some researchers consider, however, that the tricarboxylic translocase activity is virtually nonexistent in the heart and suggest that the transport of citrate occurs by mediation of the transport of 2-oxoglutarate by the dicarboxylate translocase [13,40] and the aconitase and isocitrate dehydrogenase reactions. The isocitrate dehydrogenase reaction is pH independent [41] but the correlation between the ΔpH and the citrate concentration gradient becomes dependent on the cytosolic and mitochondrial free $NADP^+$/NADPH ratios.

The transmembrane gradient of 2-oxoglutarate was 14 in the beating heart and 10 in the arrested heart. The corresponding H^+ gradients are steeper than those calculated from citrate gradients ($\Delta pH = 0.58$ and 0.50, alkaline inside, in beating and arrested hearts, respectively). This could also be interpreted as showing that citrate transport indeed occurs by the mediation of the 2-oxoglutarate transporter. If the NADP-linked isocitrate dehydrogenases catalyze equilibrium reactions both in the mitochondria and in the cytosol, these data also indicate that the poises of the free $NADP^+$/NADPH ratios are closer to each other in the cytosol and mitochondria of the heart muscle than has been previously observed in hepatocytes [34]. The $\Delta\tilde{\mu}_{H^+}$ values calculated from the membrane potentials given above and the ΔpH values derived from the 2-oxoglutarate gradient give a $\Delta\tilde{\mu}_{H^+}$ of 15.5 kJ · mol^{-1} in the beating heart and 16.2 kJ · mol^{-1} in the arrested heart. These data and the ΔG values of ATP hydrolysis as above give an H^+/ATP stoichiometry of 3.8 for the ATP synthesis in both cellular energy states studied.

It shold be pointed out that the above-described interpretation of the data rests on the principles of equilibrium thermodynamics. Although a reasonable amount of data indicate that near equilibria prevail between certain reactions of the mitochondrial respiratory chain and the extramitochondrial adenylate system in rat heart [8,30], also contradictory evidence has been presented (for references, see ref. 42). The relationship between the membrane potential and the adenylates may therefore be distorted by an amount dependent on the metabolic fluxes. However, the similar H^+/ATP ratios obtained under the two conditions studied with widely differing metabolic fluxes indicate that the error may be small.

In conclusion, the present data indicate that the cytosolic citrate concentration in intact perfused rat heart is low compared with concentrations employed in demonstrations of the citrate inhibition of glycolysis. The cytosolic citrate concentration reacts to perturbations of the mitochondrial citrate concentration, and inhibition of glycolysis at the phosphofructokinase step can be observed concomitantly with an increase in the cytosolic citrate concentration. The ΔpH across the inner mitochondrial membrane calculated from the 2-oxoglutarate concentration gradient and the mitochondrial membrane potential calculated from the adenylate distribution give an electrochemical potential difference of H^+ compatible with chemiosmotic coupling in the intact myocardium.

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