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PROTON ELECTROCHEMICAL POTENTIAL OF THE INNER MITOCHONDRIAL MEMBRANE IN ISOLATED PERFUSED RAT HEARTS, AS MEASURED BY EXOGENOUS PROBES

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The membrane potential ($\Delta\psi$) and ΔpH of the inner mitochondrial membrane were studied in isolated perfused rat hearts using exogenous labelled probes and tissue fractionation in non-aqueous media. The mitochondrial $\Delta\psi$, measured by means of the subcellular distribution of [^{3}H]triphenylmethylphosphonium ($TPMP^+$), was 125 ± 7 mV (negative inside) in hearts beating at 5 Hz and 150 ± 3 mV (negative inside) in hearts beating at 1.5 Hz. The mitochondrial membrane ΔpH, measured by means of the subcellular distribution of low concentrations of [1-^{14}C]propionate, was 0.63 ± 0.06 pH units (alkaline inside) in hearts beating at 5 Hz and 0.53 ± 0.12 pH units (alkaline inside) in hearts beating at 1.5 Hz. The implication of proton and electron gradients in the regulation of cellular respiration is discussed. In combination with previous evidence on adenylate distribution in the isolated perfused rat heart, the results indicate that the mitochondrial electrogenic adenylate translocator is in near equilibrium with $\Delta\psi$.

Introduction

A growing body of evidence exists in support of the chemiosmotic hypothesis of mitochondrial energy conservation [1,2]. Most data gathered hitherto have been obtained in experiments with suspensions of isolated mitochondria [3,4]. An experimental dilemma is the maintenance of extramitochondrial substrate concentrations at the levels encountered in vivo. These difficulties are at least in part responsible for the current controversy and conflicting results regarding the role of adenylate translocation in the regulation of cellular respiration [5–7]. Data exist of the electrical and chemical gradients of the mitochondrial membrane in isolated hepatocytes [8], but the latter are problematic in research into certain aspects of metabolism because they show signs of substrate depletion [9]. As far as the myocardium is concerned, the reproducible preparation of viable myocytes has encountered great difficulties [10].

In the present study, isolated perfused hearts were used as an intact tissue model. It has been shown previously that this tissue is amenable to the non-aqueous method of fractionation which has provided data on mitochondrial transmembrane concentration gradients [11,12]. In the present case, this method was expanded to include extrinsic probes for the mitochondrial membrane potential and pH difference. The data are compatible with chemiosmotic energy conservation in intact myocardium and also confirm previous data obtained from endogenous metabolites.

Materials and Methods

Reagents

Ordinary laboratory chemicals and triphenylmethylphosphonium bromide were obtained from

Abbreviation: $TPMP^+$, triphenylmethylphosphonium ion.

E. Merck AG, Darmstadt, F.R.G. Triphenyl-[^{3}H]methylphosphonium ([^{3}H]TPMP$^+$) iodide and [1-^{14}C]propionic acid (sodium salt) were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Propionic acid was from Fluka AG, Buchs, Switzerland. The enzymes used for the metabolite assays were obtained from Boehringer-Mannheim GmbH, Mannheim, F.R.G.

Animals and perfusion methods

Female Sprague-Dawley rats of 200–230 g from the Department's own stocks were used. No fasting period preceded the experiments. The rats were anaesthetized with intraperitoneal pentobarbital (Mebunat®, Orion Pharmaceutical Co, Helsinki, Finland), and 500 IU of heparin were injected intravenously 60 s before excision of the heart. The isolated hearts were initially perfused without recirculation by the Langendorff procedure with Krebs-Ringer bicarbonate solution [13] containing 2.5 mM $CaCl_2$ and 10 mM glucose in equilibrium with O_2/CO_2 (19:1). The perfusion pressure was 7.85 kPa (80 cm water). In some hearts the sinus node was excised, and this reduced the endogenous beating frequency to 1.2–1.5 Hz as monitored with a Statham P23 1D pressure transducer, pressure monitor and frequency meter connected to the aortic cannula, Another group of hearts was electrically paced at 5 Hz. After a preperfusion of 10 min, a recirculating perfusion with 100 ml of a medium containing 0.3–0.4 μM [^{3}H]TPMP$^+$ with a specific radioactivity of 50 000 dpm/nmol and 50 μM sodium [1-^{14}C]propionate with a specific radioactivity of 100 000 dpm/μmol was commenced and maintained for 30 min.

Oxygen consumption

Oxygen consumption was calculated from the oxygen concentration difference between the influent and effluent perfusion fluid, as measured with a Clark-type oxygen electrode (Radiometer E5046), and from the flow, as measured with a graduated cylinder.

Probe compartmentation

The heart was quick-frozen by using aluminium clamps cooled with liquid N_2 and freeze-dried at −55°C for 72 h. The tissue was homogenized and fractionated in non-aqueous media as described previously [14,11].

Solubility of the probes in the organic sovents used was tested by homogenizing a heart loaded with [^{3}H]TPMP$^+$ and [1-^{14}C]propionate to a carbon tetrachloride/heptane (1:1, v/v) mixture and incubating it for 12 h. No radioactivity was found in the solvent phase, confirming that neither the [^{3}H]TPMP$^+$ nor the sodium [1-^{14}C]propionate that had accumulated in the heart was soluble in the CCl_4/C_7H_{16} mixtures used.

When calculating the mitochondrial and cytosolic concentrations of the radioactive probes, 1.01 μl/mg total protein was taken as the amount of mitochondrial matrix water and 4.43 μl/mg total proteins as the amount of cytosolic water in the myocardium [11].

Analytical methods

Citrate synthase was used as a mitochondrial marker in the tissue fractionation and assayed as described by Shepherd and Garland [15]. Phosphoglycerate kinase was used as the cytosolic marker and assayed as described by Bücher [16]. Protein was determined by the method of Lowry et al. [17]. ADP and creatine were determined according to the method of Bernt et al. [18], and ATP and creatine phosphate as described by Lambrecht and Trautschold [19]. Inorganic phosphate was determined with glycogen phosphorylase *a*, essentially as described in Ref. 20.

Statistical treatment of the data

The significances of the differences between the means were calculated using Student's *t*-test.

Results

Heart muscle was selected as the experimental model in order to be able to alter the cellular energy consumption easily. In the present case the beating frequency was controlled by excising the sinus node. In previous studies extracellular potassium concentration changes have been used to modulate the contractility [21], but this was avoided here in order to minimize changes in the plasma membrane potential [22].

Uptake of TPMP$^+$

The uptake rate of TPMP$^+$ was rather slow, a saturating value being obtained in 20 min (Fig. 1).

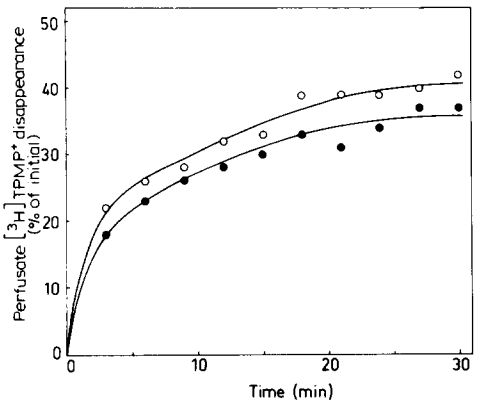

Fig. 1. Uptake of [^{3}H]TPMP$^+$ into isolated perfused rat hearts. Isolated rat hearts were perfused with recirculation by the Langendorff procedure with 100 ml Krebs-Ringer bicarbonate solution containing 10 mM glucose and 0.3–0.4 μM [^{3}H]TPMP$^+$ with a specific radioactivity of 50000 dpm/nmol. The disappearance of the radioactivity from the perfusion fluid was determined. (●) Hearts beating at 5 Hz; (○) hearts beating at 1.5 Hz. Each point represents the mean of three to five-independent determinations.

The uptake rate tended to be slightly lower in the hearts beating at 5 Hz than in those beating at 1.5 Hz. This was probably caused by the lower time-average plasma membrane potential in the hearts beating at the higher frequency.

Membrane potential

The membrane potential was calculated from the TPMP$^+$ distribution using the Nernst equation

TABLE I

PROTON AND ELECTRIC MITOCHONDRIAL TRANSMEMBRANE GRADIENTS AND METABOLITE CONCENTRATIONS IN ISOLATED PERFUSED RAT HEARTS

Isolated rat hearts were perfused as described in Materials and Methods. The membrane potentials were calculated from the distribution of [^{3}H]TPMP$^+$ and the mitochondrial transmembrane ΔpH from the distribution of [1-^{14}C]propionate, both determined by subcellular fractionation in non-aqueous media. The metabolite concentrations refer to toal concentration in the tissue. The values are means ± S.E. for the number of experiments in parentheses.

	Heart rate (Hz)	
	5	1.5
[^{3}H]TPMP$^+$ (molar ratio)		
cytosolic/extracellular	4.8 ± 0.8 (8)	4.3 ± 1.2 (6)
mitochondrial/cytosolic	109.2 ± 14.5 (8)	283.5 ± 32.2 [d] (6)
[1-^{14}C]Propionate (molar ratio)		
mitochondrial/cytosolic	4.6 ± 0.6 (8)	4.1 ± 1.0 (6)
$\Delta\psi$ (mV, negative inside)		
plasma membrane	39.6 ± 4.2 (8)	34.4 ± 6.9 (6)
mitochondrial	125.3 ± 6.6 (8)	150.1 ± 3.3 [c] (6)
ΔpH (pH units, alkaline inside)		
mitochondrial	0.63 ± 0.06 (8)	0.53 ± 0.12 (6)
$\Delta\tilde{\mu}_{H^+}$ (kJ/mol)		
mitochondrial	15.5 ± 0.5 (8)	17.1 ± 0.6 [b] (6)
O_2 consumption (μmol/min per g dry wt.)	25.9 ± 1.9 (5)	11.4 ± 0.8 [d] (3)
ATP (μmol/g dry wt.)	15.2 ± 1.0 (5)	16.3 ± 0.8 (5)
Creatine phosphate (μmol/g dry wt.)	22.6 ± 1.8 (5)	23.5 ± 1.9 (5)
Creatine (μmol/g dry wt.)	14.1 ± 1.0 (5)	11.0 ± 0.9 [a] (5)
P_i (mol/g dry wt.)	21.9 ± 3.1 (5)	14.9 ± 1.3 [a] (5)
$\frac{[ATP_f]}{[ADP_f][P_i]}$ (M^{-1}) ($\times 10^3$)	11.58 ± 2.04 (5)	22.49 ± 4.40 [a] (5)
ΔG_{ATP} (kJ/mol)	55.8 ± 0.5 (5)	57.5 ± 0.5 [a] (5)
H^+/ATP (molar ratio)	3.6	3.4

P (vs. heart beating at 5 Hz; Student's *t*-test): [a] $P < 0.05$, [b] $P < 0.025$, [c] $P < 0.01$, [d] $P < 0.005$.

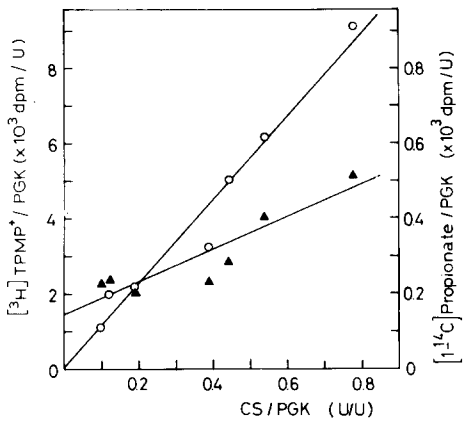

Fig. 2. A typical linear plot of the distribution of probes and of marker enzymes in the density gradient. Experimental conditions were as described in Materials and Methods. Each point represents a fraction collected from density gradient. Citrate synthase (CS) to phosphoglycerate kinase (PGK) ratio in total homogenate was 0.27 (U/U). The lines were fitted with least-squares regression. (○) [^{3}H]TPMP$^+$ ($r = 0.983$) and (▲) [1-^{14}C]propionate ($r = 0.911$).

$\Delta\psi = RT/nF \cdot \ln(C_o/C_i)$, where C_o and C_i are the outer and inner concentrations of the cation, respectively, R the gas constant, T the absolute temperature, n the charge of the cation and F the Faraday constant.

A typical tissue fractionation experiment is depicted in Fig. 2 which shows the degree of enrichment of specific tissue volumes in the density gradient fractions. Compared to the total homogenate an enrichment of the mitochondrial fraction by a factor of 3 and the cytosolic fraction by a factor of 2.7 was obtained. this is quite sufficient for calculation of the probe distribution as described in detail in Ref. 14.

The method used for the compartmental analysis allows calculation of the mitochondrial membrane potential without knowledge of the plasma membrane potential, and also determination of the latter in the same experiment.

The mitochondrial $\Delta\psi$ was dependent on the beating frequency of the heart (Table I), being lower at the higher frequency ($P < 0.01$).

The time-average $\Delta\psi$ of the plasma membrane was about 40 mV as calculated from the TPMP$^+$ distribution between perfusate and cytosol, and was not significantly different in the hearts beating at the two frequencies used.

ΔpH

The pH difference (alkaline inside) across the inner mitochondrial membrane was calculated from the distribution of a weak acid assumed to be permeable in its undissociated form, using the equation $\Delta\text{pH} = 1/n \cdot \log(A_i/A_o)$, where A_i and A_o are the concentrations of the anion in the inner and outer compartment, respectively, and n the number of dissociable protons.

Propionic acid was used as the probe because of its moderate lipophilicity and its low rate of metabolism in the myocardium. It is known that propionyl-CoA synthetase (EC 6.2.1.?) does not exist in the myocardium, where the K_m of acetyl-CoA synthetase (EC 6.2.1.1) for propionate is very high [23].

The pH across the inner mitochondrial membrane was 0.5–0.6 pH units, alkaline inside, and tended to be smaller at the lower heart frequency (Table I) although the difference was not statistically significant.

Adenylate system

The state of the free cytosolic adenylate system was estimated by measuring the tissue concentrations of creatine phosphate, creatine and P_i (Table I), assuming that the creatine kinase reaction is in near equilibrium and using the equation:

$$\frac{[ATP]_f}{[ADP]_f[P_i]} = \frac{[CrP]}{[Cr][P_i]} \cdot \frac{1}{K_{ck}}$$

where K_{ck}, the equilibrium constant of the creatine kinase reaction, is $7.058 \cdot 10^{-3}$ [24] at physiological cytosolic pH and Mg^{2+} concentration [25]. The phosphorylation potential, $[ATP]_f/[ADP]_f[P_i]$, was found to be $11.58 \pm 2.04 \cdot 10^3$ M^{-1} in the hearts beating at 5 Hz and $22.49 \pm 4.40 \cdot 10^3$ M^{-1} in those beating at 1.5 Hz ($P < 0.05$).

Correlation between proton electrochemical gradient and the ΔG of ATP hydrolysis in the cytosol

Determination of the $\Delta\psi$ and ΔpH allows the evaluation of the energy relations involved in proton translocation. The electrochemical gradient $\Delta\tilde{\mu}_{H^+} = F\Delta\psi + 2.303RT\Delta\text{pH}$ was found to be 15.5 $\pm$ 0.5 kJ/mol in the hearts beating at 5 Hz and 17.1 $\pm$ 0.6 kJ/mol in those beating at 1.5 Hz ($P <$ 0.025), and the ΔG of cytosolic ATP hydrolysis

was found to be 55.8 ± 0.51 kJ/mol at 5 Hz and 57.5 ± 0.54 kJ/mol at 1.5 Hz ($P < 0.05$) when the $\Delta G'_0$ was taken as 31.9 kJ/mol [26]. ΔG_{ATP} values calculated here by taking advantage of the creatine kinase equilibrium are very close to those obtained from compartmentspecific cytosolic concentrations of the reactants [11].

Assuming that ATP synthesis proceeds via a proton-pumping ATPase, the number of protons needed for one molecule of cytosolic ATP is equal to $\Delta G_{ATP}/\Delta\tilde{\mu}_{H^+}$, which had a value of 3.6 in the hearts beating at 5 Hz and 3.4 in those beating at 1.5 Hz. The difference was not statistically significant.

Discussion

The lipophilic cation $TPMP^+$ is in general use for the measurement of membrane potential in suspensions of mitochondria [27]. It and its analogues have also found occasional use as probes for mitochondrial or plasma membrane potential in cell suspensions [8,28,29]. The present results describe the first attempt to employ $TPMP^+$ as a membrane potential probe in intact myocardium.

Account should, perhaps, also have been taken here of the fact that the distribution gradients of $TPMP^+$ are extremely steep, and therefore are inherently prone to experimental error if the specificity of the fractionation method is not sufficiently high. An error of opposite polarity is introduced by a non-specific binding of the probe to the mitochondria. This would be difficult to test in the intact tissue used in the present investigation, however. In the light of our knowledge of the ion gradients and water volumes occupied by organelles other than mitochondria, it is probable that the contribution of nuclei [37] or cytosolic subcompartments [8] to the probe distribution is very small.

It is known that in isolated hepatocytes and fat cells the potential-independent accumulation of $TPMP^+$ in mitochondria is 12- [8] and 2.5-fold [28], respectively. Taking into account these facts, a similar degree of non-specific binding would cause an error of 3 mV maximally in this preparation, which is within the limits of experimental error.

For the use of $TPMP^+$ in non-aqueous tissue fractionation it is critical that redistribution of the probe does not occur during the fractionation. Firstly, it was found that at physiological pH, $TPMP^+$ was not soluble in the solvents used in the fractionation. Secondly, the $TPMP^+$ label was found to be associated with mitochondria in the fractionation, as could be anticipated on the basis of the mitochondrial membrane potential calculation from the endogenous substance distribution in the myocardium [11,12]. Thirdly, there is a good quantitative agreement between the membrane potential calculated from the $TPMP^+$ distribution, and that obtained previously from the ATP/ADP distribution [11,12].

The accordance between the mitochondrial $\Delta\psi$ calculated from the $TPMP^+$ distribution and that from the adenylate distribution ((both 125 mV) in the beating heart is amazing. Two conclusions can be drawn from these data: firstly, that the methods are adequate, and secondly, that it is highly probable that the electrogenic adenylate translocator [30] is in near equilibrium under physiological conditions. The principal level of control of mitochondrial oxygen consumption is still in dispute. In particular, there is no consensus as to the role of adenylate translocation in regulation. The two main hypotheses regarding the regulation of cellular respiration are the near-equilibrium hypothesis of Wilson et al. [31] and the translocase hypothesis [5,32]. The former assumes, as supported by experimental evidence, that a near-equilibrium prevails between the redox reactions of the respiratory chain and the extramitochondrial phosphorylation state of the adenylate system [21,33]. This also implies that adenylate translocation must be in near-equilibrium. The support for the translocation hypothesis under intracellular condition is mainly obtained from atractyloside inhibition studies on isolated hepatocytes [5]. Some of the reports on isolated mitochondria in support of the translocator hypothesis are open to the criticism that in order to observe a dependence of the O_2 consumption on the ADP availability, and unphysiologically low total ATP + ADP concentration is also necessary [34]. The present data give an unequivocal suggestion that under the conditions prevailing in vivo, the adenylate translocator is in near equilibrium, and the data are therefore in favour of the equilibrium hypothesis of mitochondrial respiratory control.

The mitochondrial transmembrane ΔpH values obtained using labelled propionate as a probe are in good agreement with those given by the distribution of the endogenous metabolite 2-oxoglutarate [12]. Both methods also indicate that a small decrease in the pH occurs upon an increase in the cellular energy state, even though the $\Delta\psi$ increases. Thus, the relative contribution of $\Delta\psi$ increases as the $\Delta\tilde{\mu}_{H^+}$ increases. The reason for this increase is not evident from the data.

The magnitude of $\Delta\tilde{\mu}_{H^+}$ and the relative contributions of $\Delta\psi$ and ΔpH to it are in agreement with previous data on isolated mitochodria [3]. Since data on the protonic and redox gradients and the phosphorylation state of the adenylates are now available, a unified picture of the cellular energy relations in the cardiac myocyte is emerging. The redox state of cytochrome *c* under these experimental conditions is known [21], and that of the mitochondrial concentrations of 2-oxoglutarate [12], glutamate (Kauppinen, R., Hiltunen, K. and Hassinen, I., unpublished data) and ammonia. Mitochondrial NH_3 has not been determined directly, but if we assume its distribution from the pH gradient, the glutamate dehydrogenase equilibrium yields -303 mV under these conditions. Between NAD^+ and cytochrome *c*, $\Delta G_{r/o}/\Delta\tilde{\mu}_{H^+}$ gives and $H^+/2e^-$ ratio of 3.6 for each of the phosphorylation sites. This is close to the H^+/ATP ratios observed in the present investigation (Table I). Previous evidence points to a near equilibrium in the first two phosphorylation sites [11,21]. The data on the stoicheiometry of proton pumping in the myocardium differ from the classic postulates of Mitchell [1]. Evidence is, nevertheless accumulating that the $H^+/2e^-$ stoicheiometries in each of the phosphorylation sites are higher than 2 and values close to 4 have been reported [35,36].

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