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# PLASMA MEMBRANE PHOSPHATE TRANSPORT AND EXTRACELLULAR PHOSPHATE CONCENTRATION IN THE REGULATION OF CELLULAR RESPIRATION IN ISOLATED PERFUSED RAT HEART

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**The role of extracellular $P_i$ and transmembrane fluxes across the sarcolemma in the regulation of cellular respiration was studied in isolated Langendorff-perfused rat hearts. Extracellular phosphate did not significantly affect the oxygen consumption or cellular phosphorylation potential of the myocardium. $K^+$-induced arrest was used to change the mechanical work load of the heart. Arresting the heart caused a rapid decrease in the unidirectional efflux of phosphate determined by in vitro prelabelling of the intracellular phosphate compounds with $^{32}P$ and determining the specific radioactivity of the γ-P of ATP, and the label appearance into the perfusion medium. At normal or elevated perfusate phosphate concentration there was a fairly slow net uptake of phosphate. The decrease in phosphate fluxes upon the $K^+$-induced arrest was probably not due to a decrease in the transmembrane $Na^+$ or $K^+$ gradients because a further increase in the perfusate $K^+$ concentration caused an increase in the $K^+$ efflux to the levels observed in contracting hearts. The use of higher than normal concentrations of phosphate necessitated a lowering of the extracellular $Ca^{2+}$ concentration, which caused a diminution of the oxygen consumption, accompanied by mitochondrial flavoprotein oxidation in the heart. This finding suggested that the extracellular $Ca^{2+}$ concentration may be involved in the substrate level regulation of mitochondrial metabolism.**

## Introduction

The mechanism of the adenylate control of mitochondrial respiration is still in dispute. Evidence has been presented of at least three different rate-limiting mechanisms: (1) Chemical equilibria between definite components of the mitochondrial respiratory chain and the extramitochondrial adenylate system [1,2], (2) rate-limiting adenylate translocation through the inner mitochondrial membrane [3,4] and (3) combined regulation by $P_i$ and adenylate translocation [5].

It has previously been shown that cellular respiration is regulated by the phosphorylation potential $[ATP]/[ADP] \cdot [P_i]$ in isolated pigeon heart mitochondria [6], ascites tumour cells and isolated perfused rat liver [7], isolated liver cells [8], isolated perfused rat heart [9] and *Candida utilis* cells [10].

In a tissue where the cytosolic ATP concentration is buffered by a high phosphagen content, the tissue phosphate concentration correlates well with the rate of cellular respiration [9]. On the basis of experiments on isolated perfused livers it was recently suggested that plasma membrane phosphate transport and extracellular phosphate concentration may regulate cellular respiration [11].

The plasma membrane transport of $P_i$ has previously been investigated only in a few tissues, which have shown large differences in their $P_i$-transport characteristics. In Ehrlich ascites tumour cells the

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

phosphate transport is mediated by a saturable carrier obeying Michaelis-Menten kinetics [12]. The $P_i$ distribution has been suggested to be determined by the membrane potentials [13] or active transport [14]. The discrepancy between the intracellular $P_i$ concentration calculated from an electrochemical equilibrium across the plasma membrane and the measured intracellular $P_i$ concentration has been interpreted to be caused by $P_i$ formation during the sampling procedures used [13]. The observed unidirectional $P_i$ influx in Ehrlich ascites tumour cells at 0.23 mM is 0.35 $\mu$mol/min per g $H_2O$ [12], and also in isolated perfused livers appreciable unidirectional efflux rates between 0.1 and 1.1 $\mu$mol/min per g wet wt have been measured [15], the rate being linearly dependent on the intracellular phosphate concentration. Data concerning phosphate transport in heart muscle are scarce. According to England and Walsh [16] the unidirectional $P_i$ influx in isolated perfused rat heart is a slow process.

It was recently suggested by Sestoft [11] that the plasma phosphate concentration could have a more universal significance in the regulation of oxygen consumption in vivo than has previously been thought. Therefore, it was interesting to obtain data of the role of extra- and intracellular $P_i$ concentrations and plasma membrane $P_i$ transport in the regulation of cellular respiration in myocardium. To measure the phosphate fluxes accurately, it was also necessary to devise a method for the measurement of the specific radioactivity of intracellular exchangeable phosphate. The unidirectional efflux of $P_i$ was found to depend on the mechanical work load and intracellular $P_i$ concentration. During the course of the present investigation, Medina and Illingworth [17] reported data suggesting that a mechanism involving a cotransport of $Na^+$ and $P_i$ in the plasma membrane maintains the transmembrane $P_i$ gradient, although mostly qualitative data were shown. Under all conditions tested here, the transport rates of phosphate were slow compared with the time courses of changes in the intracellular $P_i$ concentration elicited by changes in the cellular energy consumption, although the phosphate fluxes could be modulated by the external phosphate concentration and mechanical work of the heart.

## Materials and Methods

*Reagents.* The enzymes were from Sigma Chemical Co., St. Louis, MO, U.S.A., and Boehringer GmbH, Mannheim, F.R.G. Standard chemicals were obtained from E. Merck AG, Darmstadt, F.R.G. and the nucleotides and coenzymes from Boehringer GmbH.

[$^{32}$P]Orthophosphate and [$\gamma$-$^{32}$P]ATP were purchased from the Radiochemical Centre, Amersham, U.K.

*Animals.* Female Wistar rats from the stocks of the Department of Pharmacology, University of Oulu, were used, with no starvation period prior to the experiments. The rats were anaesthetized with diethyl ether and injected intravenously with 500 I.U. of heparin 1 min before excision of the heart. In the experiments where the unidirectional effluxes of $P_i$ were measured, the intracellular $P_i$ pool was prelabelled by an intraperitoneal injection of 0.5–0.8 mCi $^{32}PO_4^{3-}$ 18 h prior to the perfusion.

*Heart perfusions.* In $Ca^{2+}$-containing solutions, variation of the $P_i$ concentration is limited by the relative insolubility of $CaHPO_4$. Precipitation was avoided by decreasing the free $Ca^{2+}$ content by inclusion of EDTA, which then introduced a small free $Ca^{2+}$-buffering capacity. When studying effects of different $P_i$ concentrations, the perfusion medium was Krebs-Ringer bicarbonate solution modified to contain 2.25 mM $Ca^{2+}$, 1 mM EDTA, 10 mM glucose and 0.5, 1.2 or 3.5 mM $KH_2PO_4$. The medium was in equilibrium with $O_2/CO_2$ (19 : 1). The hearts were perfused by the procedure of Langendorff [18] without recirculation at a hydrostatic pressure of 7.84 kPa (80 $cmH_2O$). The hearts were electrically paced to a frequency of 5 Hz or arrested by increasing the KCl centration in the perfusion fluid to 18 mM and by decreasing the NaCl concentration accordingly. Oxygen concentration in the venous effluent was monitored by a Radiometer E5046 electrode and the coronary flow by means of a calibrated optical drop counter with analogue output.

The experimental protocol is depicted in Fig. 2 and included (1) a 15 min preperfusion phase with ordinary Krebs-Ringer bicarbonate solution containing 2.5 mM $Ca^{2+}$, after which (2) the perfusion was continued for 15 min with an EDTA-containing medium with an appropriate concentration of $P_i$. (3) Thereafter, the heart was arrested with KCl and the

perfusion continued for 10 min at the same $P_i$ concentration as that during phase 2.

*Surface fluorometry.* Whole organ surface fluorometry was performed with a laboratory built fluorometer [19]. For the measurement of flavin fluorescence a Farrand 465 nm interference filter and a Corning No 3 484 filter were used as the primary and secondary filters, respectively.

*Heart extracts.* Samples were obtained from the heart using aluminium tongs cooled with liquid $N_2$ [20]. The frozen pulverized sample was extracted with 8% (v/v) $HClO_4$ in 40% (v/v) ethanol precooled to −20°C [21]. Extractions was repeated with 6% (v/v) $HClO_4$ and the combined filtrates neutralized to pH 6 with 3.75 M $K_2CO_3$ containing 0.5 M triethanolamine hydrochloride.

*Metabolites.* The metabolites were determined by conventional enzymatic procedures, measuring the appearance or disappearance of NAD(P)H in an Aminco DW-2 dual-wavelength spectrophotometer using an $\epsilon_{340} - \epsilon_{385}$ value of $5.33 \cdot 10^3$ $1 \cdot mol^{-1} \cdot cm^{-1}$.

ADP and creatine were determined according to the method of Bernt et al. [22] and AMP in the same cuvette by the addition of adenylate kinase (EC 2.7.4.3). ATP was measured using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) [23] and phosphocreatine in the same assay by a subsequent addition of ADP and creatine kinase (EC 2.7.3.2). Malate was measured according to the method of Williamson and Corkey [21], glutamate with glutamate dehydrogenase (EC 1.4.1.3) [24] and aspartate with aspartate aminotransferase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37) according to the method of Bergmeyer et al. [25].

$P_i$ in the $HClO_4$ extracts was determined with glycogen phosphorylase *a* (EC 2.4.1.1), essentially as described in Ref. 26.

The adenylates, creatine, phosphocreatine and $P_i$ were measured immediately after preparation of the $HClO_4$ extract.

*Determination of $P_i$ concentration and $^{32}P$ radioactivity of $P_i$ in effluent.* The venous effluent was collected in 1-min fractions. The concentration of $P_i$ in the fractions was determined according to the method of Chen et al. [27]. To exclude the radioactivity of organic phosphate compounds, $P_i$ was extracted as phosphomolybdate into isobutanol/benzene [28] and the radioactivity determined by liquid scintillation counting using Bray's scintillator [29].

All radioactivities measured were corrected for quenching and for the decay of $^{32}P$.

*Determination of the specific radioactivity of intracellular $P_i$.* The specific radioactivity of intracellular $P_i$ was assumed to be the same as that of the γ-P of ATP because of the high rate of oxidative phosphorylation in heart muscle. The specific radioactivity of the γ-P of ATP was determined by measuring the label incorporation into histone from [γ-$^{32}$P]ATP in heart extracts by protein kinase purified from beef heart according to the method of Gilman [30].

The incubation medium [31] contained in a total volume of 0.2 ml: 10 μmol sodium glycerol phosphate buffer (pH 6.5), 40 μg histone (mixture), 2 μmol magnesium acetate, 2 μmol NaF, 0.4 μmol theophylline, 0.06 μmol EGTA, 1 nmol cyclic AMP and 0.1 ml neutralized $HClO_4$ extract of the heart. The ATP concentration in the neutralized $HClO_4$ extracts varied between 0.2 and 0.6 mM. In every incubation series at least four standards were included containing two different concentrations (0.1 and 0.2 mM) and two different specific radioactivities of the γ-P of ATP. All incubations were started by adding the protein kinase, and were performed in duplicate for 20 min at 30°C in a shaking water bath.

The reaction was terminated with 10% trichloroacetic acid, and the mixture filtered through a 0.45 μm pore-size Millipore filter. The incubation tubes were rinsed three times with 4 ml of 10% trichloroacetic acid and the washings filtered through the same filter to minimize the background radioactivity. The filters were dried and dissolved in 6 ml of ethyleneglycol monomethyl ether and the radioactivity determined in a toluene-based scintillation solution.

The $^{32}P$ radioactivity incorporation into histones was linearly proportional to the specific radioactivity of ATP and independent of the concentration of ATP in the incubation medium (Fig. 1).

*Phosphate transport.* Unidirectional $P_i$ efflux was taken as the $P_i$ radioactivity efflux divided by the specific radioactivity of intracellular $P_i$ determined as described above. Net $P_i$ influx was taken as the difference between $P_i$ concentrations in the arterial and venous perfusion fluid. Unidirectional $P_i$ influx was

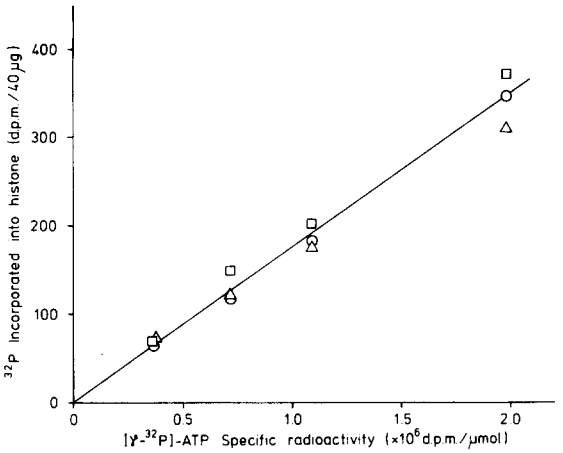


Fig. 1. Dependence of $^{32}$P incorporation into histone on the specific radioactivity of γ-P of ATP under the conditions used for the measurement of the specific radioactivity of intracellular $P_i$ in the perfused heart. Three ATP concentrations were used: (△) 0.0625 mM; (□) 0.25 mM; (○) 0.5 mM.

calculated as the sum of net influx and unidirectional efflux. The data of individual hearts were presented as averages of a 5 min period specified, determined by measurements of $P_i$ radioactivity and concentration in discrete 1 min perfusate fractions in duplicate. The data shown are means ± S.E. of a number of independent biological experiments.

*Phosphorylation potential.* The phosphorylation potential [ATP]/[ADP] · [$P_i$] was calculated from:

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

where [CrP], [Cr] and [$P_i$] are the intracellular concentrations of creatine phosphate, creatine and $P_i$, respectively, and $K_{ck}$, the pH-dependent equilibrium constant of creatine kinase, is $[MgADP^-] \cdot [CrP]/[MgATP^{2-}] \cdot [Cr] = 2.81 \cdot 10^{-10}/[H^+]$ as given in Ref. 32. At an intracellular pH of 7.4, according to $^{31}$P-NMR data [33], $K_{ck}$ amounts to $7.058 \cdot 10^{-3}$.

Extracellular water was determined in separate perfusions using inulin [$^{14}$C]carboxylic acid as an extracellular marker. Total tissue phosphate concentration was determined in neutralized $HClO_4$ extracts of the heart and converted to concentration in the total tissue water obtained as the difference between the wet and dry weights of the heart. The portion of the extracellular phosphate was calculated from the perfusate $P_i$ concentration and the inulin-accessible space of the heart representing the extracellular water (25.4 and 30.3% of total tissue water in beating and arrested heart, respectively). The intracellular $P_i$ concentration was taken as the difference between total and extracellular phosphate.

## Results and Discussion

*Oxygen consumption*

Although the primary question was whether extracellular $P_i$ has any role in the regulation of cellular respiration in the heart, the experimental design had to cope with two other interrelated parameters, viz., the extracellular calcium concentration and the mechanical work performance of the heart, and therefore due care must be taken when interpreting the data.

A decrease in the free $Ca^{2+}$ concentration in the perfusate from 2.5 to 1.25 mM decreased the oxygen consumption by 24, 29 or 18% upon a coincident change of the perfusate $P_i$ concentration from 1.18 to 0.5, 1.2 or 3.5 mM, respectively (Table I). This decreasing of the free $Ca^{2+}$ concentration by the addition of EDTA was necessary to prevent calcium phosphate precipitation when studying the higher phosphate concentrations and to stabilize the preparation at low phosphate concentrations. When the oxygen consumption rates at various phosphate concentrations in the presence of EDTA were compared, the differences found were small and not statistically significant (Table I).

In arrested hearts the effect of extracellular $P_i$ on cellular respiration could be studied without interference by differences in mechanical work performance. The oxygen consumption of $K^+$-arrested hearts was not affected by the extracellular $P_i$ concentration (Table I).

*Phosphate transport*

The unidirectional phosphate efflux was calculated from the $P_i$ radioactivity efflux and specific radioactivity of intracellular $P_i$. The unidirectional phosphate efflux in vitro from hearts prelabelled in vivo decreased rapidly upon switching to an EDTA-containing medium, irrespective of the extracellular phos-

TABLE I

EFFECT OF PERFUSATE PHOSPHATE CONCENTRATION ON INTRACELLULAR PHOSPHATE CONCENTRATION, CELLULAR ENERGY STATE AND OXYGEN CONSUMPTION OF BEATING AND ARRESTED ISOLATED PERFUSED RAT HEART

The hearts were perfused using a protocol identical to that depicted in Fig. 2 and freeze-clamped after perfusion for 15 min under sequential conditions given in the first column. The intracellular $P_i$ concentration was calculated from the intra- and extracellular water spaces and $P_i$ concentrations in the perfusate and tissue as described in Material and Methods. The values are means ± S.E. for the number of biological experiments in parentheses.

| Conditions | Intracellular $P_i$ (mM) | ATP (μmol/g dry wt.) | Phosphocreatine (μmol/g dry wt.) | Creatine (μmol/g dry wt.) | [ATP]/[ADP] · [$P_i$] ($M^{-1}$) (×$10^4$) | Oxygen consumption (μmol/min per g dry wt.) |
|---|---|---|---|---|---|---|
| 1.2 mM $P_i$ | 6.8 | 24.4 | 36.3 | 22.1 | 3.2 | 43.4 |
| 2.5 mM $Ca^{2+}$ | ± 0.2 (3) | ± 1.3 (3) | ± 1.6 (3) | ± 0.9 (3) | ± 0.2 (3) | ± 2.6 (16) |
| 0.5 mM $P_i$ | 5.1 | 23.0 | 37.2 | 20.3 | 5.2 | 33.1 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ | ± 0.3 (4) | ± 1.3 (4) | ± 1.8 (4) | ± 0.4 (4) | ± 0.5 (4) | ± 1.5 (12) |
| 0.5 mM $P_i$ | 3.0 | 22.5 | 39.2 | 18.3 | 10.1 | 14.1 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ 18 mM KCl | ± 0.1 (4) | ± 0.9 (4) | ± 0.1 (2) | ± 2.3 (2) | ± 1.0 (4) | ± 1.7 (8) |
| 1.2 mM $P_i$ | 4.2 | 24.5 | 40.4 | 21.2 | 5.8 | 31.2 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ | ± 0.8 (3) | ± 0.6 (4) | ± 3.7 (4) | ± 2.3 (4) | ± 1.3 (3) | ± 1.5 (9) |
| 1.2 mM $P_i$ | 2.9 | 23.6 | 43.3 | 17.2 | 9.5 | 15.5 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ 18 mM KCl | ± 0.4 (3) | ± 1.2 | ± 1.0 (3) | ± 3.1 (3) | ± 1.0 (2) | ± 2.6 (5) |
| 3.5 mM $P_i$ | 7.3 | 23.4 | 40.8 | 17.2 | 4.6 | 35.7 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ | ± 0.7 (3) | ± 0.9 (4) | ± 1.8 (4) | ± 0.6 (4) | ± 0.4 (3) | ± 1.3 (10) |
| 3.5 mM $P_i$ | 4.1 | 23.1 | 39.5 | 18.0 | 7.1 | 14.1 |
| 1 mM EDTA + 2.25 mM $Ca^{2+}$ 18 mM KCl | ± 0.1 (4) | ± 0.6 (4) | ± 0.7 (4) | ± 0.6 (3) | ± 0.6 (4) | ± 1.5 (6) |

phate concentration (Table II). This decrease was more prominent at high phosphate concentrations, but slow recovery towards the initial flux values always occurred during the following 15 min. When the mechanical work load was eliminated by high-$K^+$ arrest of the heart, a decrease in the $P_i$ efflux was very reproducibly observed. Under these conditions, an inverse correlation between the perfusate $P_i$ concentration and unidirectional $P_i$ efflux was also found (Fig. 2, Table II).

During the course of the present investigation, Medina and Illingworth [17] reported data on the phosphate transport in perfused hearts. Although quantitative data were not reported, their efflux data were qualitatively similar to ours. It is noteworthy that in the perfused heart preparation of Medina and Illingworth [17], lowering of the extracellular $K^+$ concentration resulted in an increase in the $P_i$ efflux, although an increase in the extracellular $K^+$ concentration failed to produce the reciprocal decrease unless high enough concentrations were used to arrest the heart. Therefore, the $K^+$-induced changes in $P_i$ transport observed here may be due more to phenomena related to muscle contraction than to changes in the cation gradients across the plasma membrane. An increase in the $K^+$ concentration from 4.7 to 18 mM decreases the resting membrane potential from −80 to −55 mV [34], but the most striking effect of 18 mM $K^+$ is the abolition of the action potentials and the pacemaker activity [35], so that the membrane potential is stabilized at the resting potential. Experiments on plasma membrane vesicles isolated from mouse fibroblasts have shown that the transmembrane $P_i$ transport is energized by an electrochemical $Na^+$ gradient and that the $P_i$ anion in these membranes moves in cotransport with $Na^+$ [36]. In the

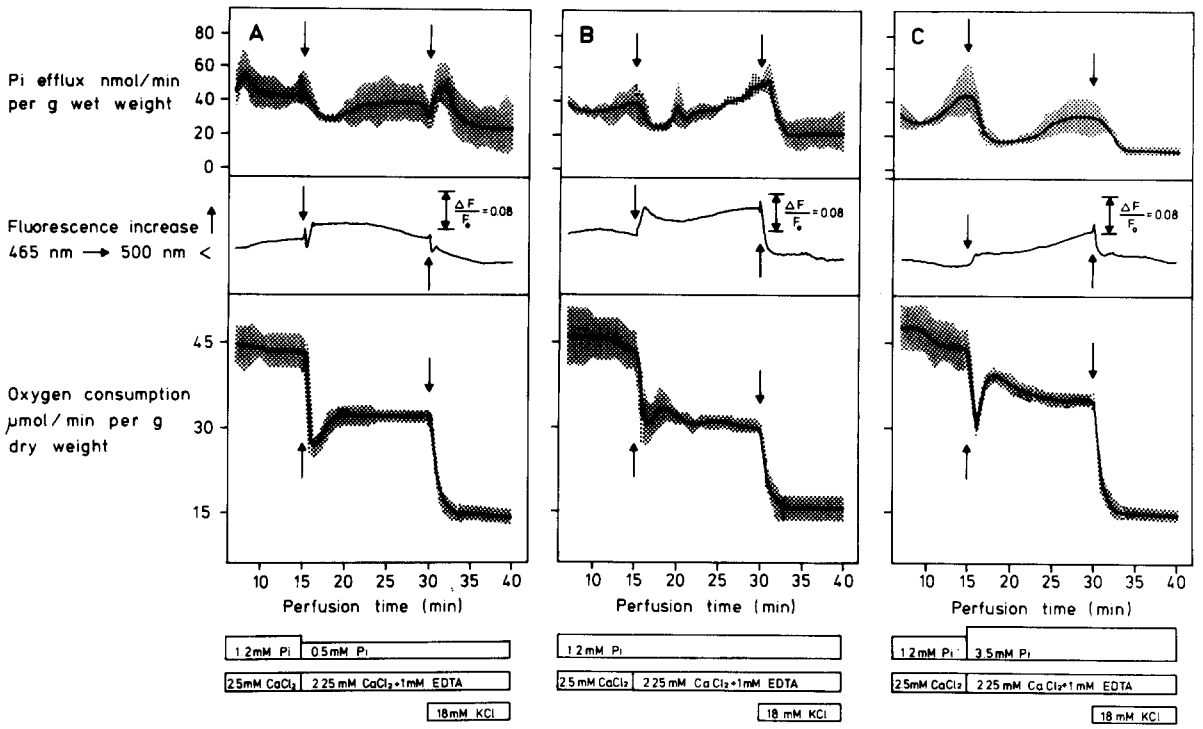


Fig. 2. Effect of changes in the perfusate $Ca^{2+}$ and $P_i$ concentration on the unidirectional $P_i$ efflux, flavoprotein redox state and oxygen consumption in isolated perfused rat hearts. In the upper panel the curve is the mean of three and in the lower panel of 5–12 independent experiments. The shaded area represents ± S.E. The middle panel depicts typical tracings of epicardial flavoprotein fluorescence. Increase in the fluorescence above 500 nm indicates flavoprotein oxidation.

TABLE II

EFFECT OF PERFUSATE COMPOSITION ON THE PHOSPHATE FLUXES ACROSS THE PLASMA MEMBRANE IN BEATING AND ARRESTED PERFUSED RAT HEARTS

The intracellular phosphates were labelled in vivo with $^{32}PO_4^{3-}$, and the label efflux and net flux of $P_i$ determined. The label efflux was converted to moles of phosphate transported by determining the specific radioactivity of ATP which was taken to represent the specific radioactivity of intracellular $P_i$. The average fluxes were measured for the 5-min periods 10–15 min, 25–30 min and 35–40 min for the conditions a, b and c, respectively. The values are the means ± S.E. for the number of independent biological experiments in parentheses and are expressed as nmol/min per g wet wt. See Fig. 2 for the experimental protocol and timing.

| Perfusion conditions | Unidirectional efflux | Unidirectional influx | Net influx |
|---|---|---|---|
| Expt. 1 | | | |
| (a) 1.2 mM $P_i$, 2.5 mM $Ca^{2+}$ | 43.0 ± 7.4 (3) | 199 ± 25 (3) | 152 ± 20 (3) |
| (b) 0.5 mM $P_i$, 1 mM EDTA, 2.25 mM $Ca^{2+}$ | 37.8 ± 10.8 (3) | 8.0 ± 3.9 (3) | −29.7 ± 7.1 (3) |
| (c) 0.5 mM $P_i$, 1 mM EDTA, 2.25 mM $Ca^{2+}$, 18 mM KCl | 27.3 ± 11.9 (3) | 3.7 ± 0.9 (3) | −26.5 ± 9.4 (3) |
| Expt. 2 | | | |
| (a) 1.2 mM $P_i$, 2.5 mM $Ca^{2+}$ | 34.1 ± 5.1 (3) | 156 ± 7 (3) | 122 ± 5 (3) |
| (b) 1.2 mM $P_i$, 2.25 mM $Ca^{2+}$, 1 mM EDTA | 42.8 ± 3.9 (3) | 126 ± 17 (3) | 83.9 ± 20.3 (3) |
| (c) 1.2 mM $P_i$, 1 mM EDTA, 2.25 mM $Ca^{2+}$, 18 mM KCl | 21.4 ± 10.0 (3) | 41.7 ± 6.4 (3) | 20.3 ± 7.3 (3) |
| Expt. 3 | | | |
| (a) 1.2 mM $P_i$, 2.5 mM $Ca^{2+}$ | 34.2 ± 4.9 (3) | 174 ± 16 (3) | 142 ± 11 (3) |
| (b) 3.5 mM $P_i$, 1 mM EDTA, 2.25 mM $Ca^{2+}$ | 26.5 ± 8.1 (3) | 168 ± 13 (3) | 142 ± 6 (3) |
| (c) 3.5 mM $P_i$, 1 mM EDTA, 18 mM KCl, 2.25 mM $Ca^{2+}$ | 12.2 ± 2.8 (3) | 94.2 ± 7.5 (3) | 80.6 ± 4.8 (3) |

same experimental model evidence of both $Na^+$-dependent and $Na^+$-independent $P_i$ transport has been obtained [37].

It was suggested by Medina and Illingworth [17] that the $P_i$ flux is dependent on the transmembrane $Na^+$ gradient and is mediated by an electroneutral cotransport of $Na^+$ and $P_i$. When the extracellular $K^+$ concentration is increased at the expense of $Na^+$, effects on the $Na^+$ gradient may be compensated for by an activation of the $(Na^+ + K^+)$-stimulated ATPase by high $[K^+]$, and if a coupling between $Na^+$ and $P_i$ transport exists, $P_i$ fluxes should also increase. The effect of $Na^+$ and $K^+$ gradients on the $P_i$ transport was studied in arrested hearts by further increasing the $K^+$ concentration above 18 mM. When the perfusate $K^+$ concentration was 24 mM, the unidirectional $P_i$ efflux increased to a value twice that in the presence of 18 mM $K^+$. When the $K^+$ concentration was increased to 30 mM, the $P_i$ efflux remained at the same level as that in the presence of 24 mM. The data showed that under conditions allowing for high activity of the $(Na^+ + K^+)$-stimulated ATPase, the unidirectional $P_i$ fluxes at the plasma membrane are also high. This could be explained by a connection between $Na^+$ and $P_i$ transport.

The $Na^+$ conductivity of the heart muscle cell plasma membrane has been experimentally determined, and in perfused dog heart papillary muscle it amounts to 5 pmol/s per $cm^2$, which is equivalent to 0.28 $\mu$mol/min per g wet wt. when taking the plasma membrane area as 930 $cm^2$/g myocardial wet wt. [38]. When a stoichiometry of three $Na^+$ to one ATP is used for the $(Na^+ + K^+)$-activated ATPase [39], the ATP consumption due to $Na^+$ pumping is 93 nmol/min per g wet wt. which at a P/O ratio of 3 amounts to 1.2% of the oxygen consumption. Using a tentative $Na^+/P_i$ stoichiometry of 2 for the cotransport of $Na^+$ and $P_i$, the inward flux of phosphate would be 140 nmol/min per g wet wt., which is somewhat higher than the 41.7 nmol/min per g wet wt. observed in the present study in arrested hearts perfused with 1.2 mM phosphate (Table II).

In a beating heart the $Na^+$ flux has been estimated to be 13.5 pmol/$cm^2$ (12.6 nmol/g wet wt.) per excitation [40], which, added to the resting flux, totals 4.06 $\mu$mol/min per g wet wt. at a frequency of 5 Hz, equivalent to 1.35 $\mu$mol ATP/min per g wet wt. and accounting for 5.1% of the oxygen consumption. Thus, the $P_i$ influx estimated from the $Na^+$ fluxes in the beating heart is 2.03 $\mu$mol/min per g wet wt. as against the 0.156 $\mu$mol/min per g wet wt. measured (Table II). It must, however, be borne in mind that there is evidence that in short-term experiments the rate of $Na^+$ pumping at the plasma membrane does not necessarily closely follow a change in the inward flux, but a considerable lag period may ensue [41]. The present results, as well as those of Medina and Illingworth [17], demonstrate a positive correlation between the phosphate fluxes and the mechanical work load in the heart. Comparison of the measured phosphate fluxes with the resting $Na^+$ transport estimated on the basis of electrophysiological data is in accord with the notion of a cotransport of $Na^+$ and $P_i$. On the other hand, in the contracting heart, the $P_i$ fluxes calculated from the electrophysiological data differ from the measured $P_i$ fluxes by a factor of more than 10.

The unidirectional $P_i$ influx was calculated from the measured $P_i$ efflux and net $P_i$ flux. The latter was determined from the arteriovenous $P_i$ concentration difference and coronary flow. There was a very small net flux of phosphate inwards, when the perfusate $P_i$ concentration was 1.2 or 3.5 mM. Only at an external $P_i$ concentration of 0.5 mM did a net outward flux exist. In this context it is noteworthy that in perfused livers it has been shown that the unidirectional $P_i$ efflux is proportional to external $P_i$ concentration, possibly because of modulation of the intracellular $P_i$ concentration, which is the main determinant of the unidirectional $P_i$ efflux in liver [15]. In the present study, no effects of the external $P_i$ concentration on the intracellular $P_i$ concentration could be demonstrated, the latter being mainly determined by the cellular energy state. The present results also emphasize that large differences in the phosphate transport capacity of the plasma membrane exist between various tissues.

*Intracellular $P_i$ and high-energy phosphate content*

Compared with heart perfused with ordinary Krebs-Ringer bicarbonate solution containing 2.5 mM $Ca^{2+}$ and 1.18 mM $P_i$, the intracellular $P_i$ concentration after changing to an EDTA-containing medium with 1.25 mM free $Ca^{2+}$ was low, irrespective of the extracellular concentration of $P_i$. Since the phosphocreatine concentration increased simultaneously, the

changes in the $P_i$ concentration were obviously due to changes in the cellular energy state.

Arresting the heart with the high-$K^+$ medium caused a further decrease in the intracellular $P_i$ concentration with a concomitant increase in the phosphocreatine concentration.

The net rates of $P_i$ transport observed are sufficient to have a measurable effect of the intracellular $P_i$ concentration, and the balance of phosphagen plus $P_i$ changes and the corresponding measured rates of $P_i$ transport are in good agreement within the limits of experimental error.

*Cellular redox state*

Mitochondrial redox changes were studied in an attempt to define the level of metabolic control expressed in the changes in the oxygen consumption. Tissue flavoprotein fluorescence, previously identified as originating from lipoate dehydrogenase [42], was used as a qualitative indicator of the mitochondrial NADH/NAD ratio. Upon the change to perfusion with the EDTA-containing low-$Ca^{2+}$ medium, an oxidation of the flavoproteins was observed. This response was not modified by changes in the perfusate $P_i$ concentration. The flavoproteins were always reduced upon a $K^+$-induced arrest of the heart, in accord with previous observations [9,19].

The flavoprotein oxidation which was constantly observed upon lowering the extracellular $Ca^{2+}$ concentration is difficult to explain in the light of the current knowledge of regulation of mitochondrial terminal oxidations. Because an inhibition of the mitochondrial respiratory chain should result in an $NAD^+$ (and flavoprotein) reduction, the paradoxical mitochondrial NADH oxidation can only be explained by a lowered hydrogen pressure in the mitochondrial $NAD^+$ pool due to substrate-level regulation.

It is known that some of the rat heart mitochondrial enzymes, $NAD^+$-linked isocitrate dehydrogenase [43], 2-oxoglutarate dehydrogenase [44] and pyruvate dehydrogenase phosphatase [45] are activated by $Ca^{2+}$, and by this means, substrate-level regulation of mitochondrial NADH production by the intramitochondrial $Ca^{2+}$ concentration is possible. The redox changes elicited in the mitochondria in situ by extracellular $Ca^{2+}$ therefore could be interpreted to show that although the average cytosolic free $Ca^{2+}$ concentration is extremely low, the mitochondrial $Ca^{2+}$ concentration is somehow linked with the extracellular free $Ca^{2+}$ concentration, which then might have a role in the regulation of mitochondrial metabolism.

## Conclusions

In isolated perfused hearts the extracellular phosphate concentration has little effect on the cellular respiration, although a slow net inwards flux of phosphate was observed at normal or elevated perfusate phosphate concentrations. However, as demonstrated in $K^+$-arrested hearts, mechanical work of the heart influences the unidirectional efflux rate of phosphate. The decrease observed in the phosphate efflux upon the $K^+$-induced arrest is probably not due to changes in the transmembrane gradients of $K^+$ and $Na^+$. It is more likely that the phosphate fluxes may be influenced by the rate of $Na^+$ pumping, which changes according to the $Na^+$ fluxes contributed by the electrophysiological phenomena associated with the initiation of muscular contraction.

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