Some Factors Affecting Phosphate Transport in a Perfused Rat Heart Preparation

Gloria MEDINA and John ILLINGWORTH

Department of Biochemistry, University of Leeds, Leeds LS2 9LS, U.K.

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P_i uptake by a perfused rat heart preparation did not require the presence of any other permeant anion, but was markedly dependent on the extracellular Na^+ concentration and accelerated when tissue oxygenation was inadequate. P_i efflux was also independent of other permeant anions, but apparently varied with the intracellular Na^+ concentration. Cardiac P_i efflux was not sensitive to a number of inhibitors that block Cl^- movement in heart and other tissues. Both uptake and efflux apparently proceed via a reversible electroneutral co-transport system linked to the transmembrane Na^+ gradient. P_i uptake was independent of cardiac work load, but the efflux rate was sharply accelerated after an increase in aortic pressure development, with a slow return towards basal values during sustained periods of high work output. An inverted biphasic effect on the efflux rate was observed after a reduction in cardiac work load. Mild hypoxia and respiratory and metabolic acidosis each resulted in a transient acceleration of P_i efflux followed by a return towards basal values during prolonged exposure to the stimulus, whereas respiratory and metabolic alkalosis produced a similar but inverted response. The origin of these phasic effects on P_i efflux remains to be identified at present.

In recent years P_i has been recognized as a major regulator of intracellular metabolism. The P_i concentration directly affects the activity of a number of important enzymes, including glycogen phosphorylase (Wollenberger & Krause, 1968; Graves & Wang, 1972; Neely & Morgan, 1974), glyceraldehyde phosphate dehydrogenase (Harris & Waters, 1976), succinyl-CoA synthetase (Bridger, 1974) and the mitochondrial F_1 -ATPase (Schuster *et al.*, 1977; Kayalar *et al.*, 1976), and, in addition, the cytoplasmic phosphate potential $\{[ATP]/([ADP] \times [P_i])\}$ has been shown to play a central role in the integration of cellular energy metabolism (Erecinska *et al.*, 1978).

The concentration of P_i in cardiac muscle has been shown to vary with work load (Neely *et al.*, 1970; Opie *et al.*, 1971; Illingworth *et al.*, 1975) and also increases when tissue oxygenation becomes inadequate (Opie *et al.*, 1972; Mathur & Case, 1973) as a result of the breakdown of ATP and phosphocreatine (Williamson, 1966; Neely *et al.*, 1973). Under at least some sets of circumstances cellular P_i concentrations remain elevated for several

hours (Fleckenstein, 1971), despite the existence of a P_i -transport system in the cell membrane, suggesting that cardiac muscle, and perhaps other tissues, have the ability to regulate their internal P_i content according to the needs of the organism.

P_i must be accumulated by heart and skeletal muscle against a considerable electrochemical gradient, since under normal conditions the Nernst potential for $H_2PO_4^-$ (about +40 mV) differs markedly from the resting membrane potential of about -80 mV. This uptake might either be driven directly by the hydrolysis of ATP, or might alternatively result from the co-transport of P_i with other ions for which there is a pre-existing transmembrane concentration gradient. It is most unlikely, however, that P_i movements across the plasmalemma could be driven by a transmembrane proton gradient by a transport system analogous to the mitochondrial P_i carrier, since the plasmalemma transmembrane pH gradient is orientated in the wrong direction for P_i uptake.

This paper seeks to clarify the mechanism of P_i accumulation in cardiac muscle, and suggests that, as in other tissues, P_i uptake and efflux are coupled to the electroneutral co-transport of Na^+ ions, although there must be additional factors that also influence the rate of P_i migration.

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid.

Materials and Methods

[^{32}P]Orthophosphate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Enzymes and substrates for metabolite assays were supplied by either Sigma (London) Ltd. or by Boehringer Corp., and all other laboratory reagents were of recognized analytical grade. Furosemide was obtained in the form of 'Lasix' injections (Hoechst Pharmaceuticals), and Verapamil in the form of 'Cordilox' injections (Abbott Laboratories, Queenborough, Kent, U.K.). *N*-Ethylmaleimide and 4-acetamide-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) were obtained from BDH. Mersalyl and 5,5'-dithiobisnitrobenzoic acid were obtained from Sigma (London) Ltd.

The rat heart perfusion technique originally described by Neely *et al.* (1967) was extensively modified for these experiments in order to provide improved control of left-atrial filling pressure and aortic afterload (Illingworth *et al.*, 1975; Illingworth & Mullings, 1976a). Male Sprague-Dawley rats (approx. 500 g) were killed with a guillotine decapitator without prior use of anaesthetics or anticoagulants, and the hearts perfused at 35°C with Krebs bicarbonate Ringer or with a variety of other solutions as described in Table 1. All hearts were electrically paced at 5 beats/s, unless otherwise stated, and were assessed for maximal work output [normally at least 17.3 kPa (130 mmHg) with closed aorta and unrestricted atrial filling] before the start of an experiment.

For P_i -uptake experiments, each heart was normally equilibrated for 5 min at the desired aortic pressure before infusion of a stock solution of radioactive P_i made up in perfusion medium at about 5% of the total coronary flow rate into either the atrial-filling cannula (working-heart experiments) or the aortic cannula (Langendorff preparations). We found it essential to filter the stock P_i solution through a 0.22 μm Millipore filter (type Millex-GS) during the infusion in order to avoid artificially high P_i uptakes apparently associated with the adsorption of radio-label on particulate contaminants in the perfusion buffer, which subsequently became trapped within the tissue. Labelling normally continued for 10 min, after which the heart was flushed for a further 5 min with non-radioactive buffer in order to purge the cannulae and the vascular and interstitial spaces before the organ was freeze-clamped for subsequent tissue extraction and determination.

As plasmalemma P_i transport is relatively slow in cardiac muscle in relation to the size of the intracellular pools (see the Results and Discussion section), the intracellular specific radioactivity of $^{32}\text{P}_i$ never approached equilibrium with the extracellular label in our experiments. Losses of $^{32}\text{P}_i$ from the intracellular pools of exchangeable phosphate com-

Table 1. Ionic compositions of perfusion buffers

All solutions contained 1.2 mM-Mg²⁺, 2.5 mM-Ca²⁺ and 1 mM-sodium pyruvate or 0.5 mM-octanoate. All Hepes, Mes and triethanolamine buffered solutions were adjusted to pH 7.4 (35°C) with 1 M-NaOH after equilibration with 100% O₂.

Buffer	Ringer name	Ionic composition (mM)										Gas phase (%)			
		Na ⁺	K ⁺	Cl ⁻	SO ₄ ²⁻	PO ₄ ³⁻	HCO ₃ ⁻	Hepes	Isethionate	NO ₃ ⁻	Acetate	Triethanolamine	Mes	O ₂	CO ₂
A	Krebs bicarbonate	145	5.6	130	1.2	0.6	25	—	—	—	—	—	—	95	5
B	Hepes Ringer	142.5	5.6	150	1.2	0.6	—	5	—	—	—	—	—	100	—
C	Isethionate/Hepes	142.5	5.0	—	—	—	—	5	152.4	—	—	—	—	100	—
D	Chloride/Hepes	142.5	5.0	152.4	—	—	—	5	—	—	—	—	—	100	—
E	Mes Ringer	142.5	5.0	—	—	—	—	—	—	—	—	—	157.4	100	—
I	Isethionate/bicarbonate (only Cl ⁻ replaced)	145	5.6	5	1.2	0.6	25	—	125	—	—	—	—	95	5
J	Acetate/bicarbonate (only Cl ⁻ replaced)	145	5.6	5	1.2	0.6	25	—	—	—	125	—	—	95	5
K	Triethanolamine Ringer	138.2	5.6	155	1.2	0.6	—	—	—	—	—	10	—	100	—

pounds during the combined labelling and rinsing periods were estimated to be less than 10% of the accumulated radioactivity. No correction has been made for this error in the results presented here. Loading buffers containing a final radioactive concentration of $200 \mu\text{Ci } ^{32}\text{P}_i/\text{litre}$ gave convenient uptakes of about $0.4 \mu\text{Ci}$ of $^{32}\text{P}_i$ per heart during a 10 min labelling period with 0.6 mM extracellular P_i . Lower radioactive concentrations were used in several of our experiments, and where appropriate this has been indicated in the Figure legends.

The frozen hearts were finely powdered under liquid N_2 , and extracted with 5% (v/v) HClO_4 by established methods (Williamson & Corkey, 1969). P_i was determined immediately on the deproteinized tissue extract without neutralization by a slight modification of the solvent extraction method described by Wähler & Wollenberger (1958). Fluorimetric and spectrophotometric metabolite measurements were made on the neutralized tissue extracts by the methods of Williamson & Corkey (1969), Lowry & Passoneau (1972) and Bergmeyer (1978). The tissue dry weights were determined by drying the HClO_4 -precipitated pellet to constant weight as described by Illingworth *et al.* (1975).

Hearts were severely vasoconstricted after prolonged exposure to low- Na^+ Ringer, so for these experiments $^{32}\text{P}_i$ uptake was assessed in Langendorff preparations with a Geiger counter securely clamped 2.5 cm from the heart after 1 min exposure to the test solution containing $^{32}\text{P}_i$ followed by 5 min flushing with normal- Na^+ Ringer. The Geiger tube was shielded from radioactive splashes by a thin plastic screen, and the outside of the heart was briefly rinsed with Ringer during each flushing period. We found it helpful to protect the Geiger tube from light and also from radiation emitted by pipework containing perfusion buffer in order to achieve low background count rates. In this way it was possible to perform a series of uptake measurements on each heart, and directly compare uptake in low- Na^+ solutions with that observed in alternating exposures to a control Ringer containing a normal Na^+ concentration and $^{32}\text{P}_i$ of identical specific radioactivity.

For studies on P_i efflux we normally labelled the hearts in Krebs bicarbonate Ringer, or in Hepes Ringer (buffer B, Table 1) when the efflux measurements involved non-bicarbonate-buffered solutions. The radioactive stock solution was infused for 30 min, followed by at least 5 min flushing with non-radioactive buffer before effluent samples were collected for Cerenkov counting. In later experiments we found it advantageous to wait for up to 15 min before collecting samples, since the increased opportunity for label equilibration within the intracardiac phosphate compounds then seemed to result in a flatter baseline for the washout graphs.

The whole of the coronary effluent was normally collected directly into plastic scintillation vials, and we varied the collection intervals from 30 to 60 s according to the coronary flow rate, so as to achieve a sample volume of between 8 and 16 ml.

Results and Discussion

Uptake measurements (Fig. 1) over a range of external P_i concentration revealed a saturable process with an apparent K_m of 0.24 mM in working hearts perfused at either high work loads [17.3 kPa

Fig. 1. Effect of the extracellular concentration of P_i on $^{32}\text{P}_i$ uptake

Working-heart preparations were equilibrated for 5 min in Krebs bicarbonate Ringer of the particular potassium orthophosphate concentration. Stock $^{32}\text{P}_i$ -containing Ringer ($0.5 \mu\text{Ci}$ of P_i/ml) was injected for 10 min into the atrial-filling line, at 5% of the coronary flow, with the help of a peristaltic pump. High-work-output preparations were perfused at an unrestricted atrial-filling rate. Low-work-output hearts were continuously perfused at 5.3 kPa (40 mmHg). The hearts were freeze-clamped after a 5 min rinsing period. Net $^{32}\text{P}_i$ uptake during 10 min was calculated from the ^{32}P radioactivity present in 1 g of dry tissue divided by the specific radioactivity of extracellular $^{32}\text{P}_i$, determined on the pooled coronary effluent. Lineweaver-Burk plots of the data are shown. Each point is the mean value of the results obtained for three hearts. (a) Hearts working at 17.3 kPa (130 mmHg) mean aortic pressure; (b) hearts working at 5.3 kPa (40 mmHg) mean aortic pressure.

(130 mmHg) aortic pressure] or at low work outputs [5.3 kPa (40 mmHg) aortic pressure]. It would appear that the uptake mechanism is almost saturated by physiological external P_i concentrations. Since the system appeared to be almost independent of work load, the majority of the remaining experiments were performed on working hearts at a standard [13.3 kPa (100 mmHg)] aortic pressure. Net uptake was somewhat depressed after prolonged exposure to $^{32}P_i$ (Fig. 2) as a result of efflux during labelling, but it did not vary significantly when we altered the equilibration time with non-radioactive P_i , or when we changed the perfusion temperature by $\pm 5^\circ C$ (Table 2). Mild hypoxia, however [buffer gassed with $O_2/N_2/CO_2$ (57:38:5)], resulted in a marked enhancement of uptake, from $4.42 \pm 0.34 \mu\text{mol}$ of P_i/g dry wt. ($n=14$) in 'normoxic' hearts to $19 \pm 1.77 \mu\text{mol}$ of P_i/g dry wt. ($n=3$) under hypoxic conditions. The tissue P_i content was also markedly elevated under these conditions, from 9.63 ± 0.6 to $14.19 \pm 0.34 \mu\text{mol}$ of P_i/g dry wt. (means $\pm$ S.E.M.). We had some indication that P_i -uptake rate varied with pacing speed, though this proved a difficult variable to study and we decided merely to keep the beat frequency as constant as possible.

In erythrocytes, where anion translocation apparently proceeds without any energy input, P_i , SO_4^{2-} , Cl^- and HCO_3^- seem to share a common carrier mechanism and compete for uptake (Cabantchik *et al.*, 1978; Fortes, 1977). The K_m for

P_i may be as high as 80 mm in this system (Ho & Guidotti, 1975) and Cl^- and HCO_3^- are the preferred substrates. In contrast with this, we found that in heart muscle replacement of almost all of the Cl^- with the non-permeant ion isethionate (hydroxyethanesulphonate), or by acetate, had very little effect on P_i -uptake rates, neither was there significant change in bicarbonate-free Ringer (see Fig. 3a). Analyses of the frozen hearts revealed that tissue ATP, phosphocreatine and P_i remained essentially normal after 15 min perfusion with these nonphysiological media (see Fig. 3b). Acetic acid penetrates rapidly through most biological membranes, and apparently equilibrates across the cardiac sarcolemma. Acetate anion is only slowly permeant in muscle cells (Woodbury & Miles, 1973) and as a result the internal acetate concentration is governed by the external acetate concentration and the transmembrane pH gradient. Under our conditions, this internal acetate pool leads to an approx. 30% increase in cell volume (G. Medina & J. Illingworth, unpublished observations) without seriously affecting cardiac performance. We conclude that it is possible to make gross alterations to both intracellular and extracellular anion compositions without significantly altering P_i uptake, and these results clearly indicate that the cardiac P_i porter differs fundamentally from the erythrocyte system, and suggest that P_i uptake does not take

Fig. 2. Time course of $^{32}P_i$ uptake

Langendorff preparations were perfused with Krebs bicarbonate Ringer at 13.3 kPa (100 mmHg) mean aortic pressure. After the 5 min equilibration period the hearts were labelled at random for different lengths of time, with ^{32}P -containing medium (approx. $100 \mu\text{Ci/litre}$) as indicated in the Figure. The preparations were freeze-clamped after 5 min rinsing with non-radioactive buffer. P_i uptake was calculated as for Fig. 1. The data represent the means $\pm$ S.E.M. for four independent observations at each time point.

Table 2. Phosphate uptake and perfusion conditions

Cannulation required approx. 10 min, after which the hearts were equilibrated as working-heart preparations at 13.3 kPa (100 mmHg) mean aortic pressure with Krebs bicarbonate Ringer (0.6 mM- P_i) before the radioactive isotope was infused. The data come from two experiments. In the first series of hearts the equilibration period was varied and in the second one, the perfusate temperature was 30, 35 or $40^\circ C$. All hearts were perfused for 10 min with medium containing $^{32}P_i$ and were rinsed for 5 min with non-radioactive buffer before being freeze-clamped. Net P_i uptake was calculated as described in the legend to Fig. 1. Results are means $\pm$ S.E.M. for the numbers of determinations indicated in parentheses.

Perfusate temperature ($^\circ C$)	Equilibration period (min)	Net uptake in 10 min (μmol of P_i/g dry wt.)
35	3	2.22 and 4.24 (2)
35	5	4.45 ± 0.78 (3)
35	10	4.88 ± 0.92 (3)
35	15	4.17 ± 0.85 (3)
35	20	4.97 ± 0.36 (3)
30	5	3.55 ± 0.12 (3)
35	5	4.53 ± 4.84 (2)
40	5	4.33 ± 0.18 (3)
Total mean value ...		4.31 ± 0.22 (22)

Fig. 3. Effects of Cl^- and HCO_3^- -free perfusions on $^{32}\text{P}_i$ uptake

Hearts were perfused as working preparations at 13.3 kPa (100 mmHg) aortic pressure. The hearts were initially cannulated and assessed in Krebs bicarbonate Ringer before equilibration and labelling in the test solution as described in the legend to Fig. 1. The ionic composition of the buffers is reported in Table 1. (a) Effect on $^{32}\text{P}_i$ uptake. (b) Content of phosphates in the hearts perfused for 15 min with the solutions indicated. Intracellular P_i concentration was calculated from the total weight of dry tissue, a value of intracellular volume of 2.46 g/g of dry rat ventricle (Polimeni, 1974; G. Medina & J. Illingworth, unpublished observations) and the P_i concentration determined on the perfusate and on the tissue. Results are means $\pm$ S.E.M. for five determinations. ■, P_i ; □, ATP; ▨, phosphocreatine.

place by reciprocal exchange with either Cl^- or HCO_3^- .

P_i transport is known to be coupled to the movement of Na^+ ions in many other tissues, including mammalian renal and intestinal epithelia (Berner *et al.*, 1976; Hoffman *et al.*, 1976; Kinne *et al.*, 1977), toad urinary bladder (Sellers *et al.*, 1978), chick duodenum (Peterlik, 1978), fish and mammalian nerve fibre (Straub *et al.*, 1976, 1977; Ferrero *et al.*, 1978; Anner *et al.*, 1976), ascites cells (Brown & Lamb, 1975; Scholnick *et al.*, 1973) and in fibroblasts (Lever, 1978; Hamilton & Nielsen-Hamilton, 1978). It would therefore not be surprising if cardiac P_i uptake were to proceed by a

similar mechanism, and our own experiments seem to confirm this hypothesis. In the experiments with the externally placed Geiger counter, the average uptake after 1 min exposure to $^{32}\text{P}_i$ in Krebs bicarbonate Ringer was 6.25 ± 0.5 arbitrary units ($n = 4$), whereas in low- Na^+ Ringer (100 mM-choline chloride replaces the same proportion of NaCl in Krebs bicarbonate Ringer), plus 100 mg of atropine/litre, the uptake averaged 3.5 ± 0.31 arbitrary units ($n = 4$) (see Fig. 4).

P_i efflux from the heart follows a quasi-exponential decay curve once radioactive material has been largely flushed from the vascular and interstitial spaces approx. 3 min after the cessation of labelling. Preliminary experiments confirmed that the bulk of the labelled material leaving the heart was indeed P_i , in agreement with the results of Anner *et al.* (1976), who have studied flows of $^{32}\text{P}_i$ in nerve cells.

The efflux data presented in Figs. 5–13 consist of representative traces from individual hearts. Each experiment was normally repeated at least three times. The overall response patterns were very reproducible, but it proved difficult to average the efflux data because the effluent count rates and washout speeds differed appreciably from one heart to the next. We doubt that this reflects any excessive variation in transport rate, since small heart-to-heart differences in transport velocity will affect both the amount of label loaded and the fractional efflux rate, leading to a substantial combined effect on the washout curve.

Somewhat to our surprise, we observed a biphasic response when hearts were challenged with a work jump from 6.7 to 20 kPa (50 to 150 mmHg) aortic pressure (Fig. 5). The initial rapid increase in efflux was followed by a slow return towards the basal value, even though the work challenge was continued. Furthermore, it will be noted that an inverted response was produced on return to the lower work output, with a steep decline followed by a slow recovery to normal values. The experiment could be repeated, but on subsequent trials the effect became progressively smaller. Some of the increased efflux might be attributable to the elevated tissue P_i concentrations after a work challenge (Illingworth *et al.*, 1975), but this is not adequate to explain the size of the initial transient increase, nor does it account for the subsequent decline, since tissue P_i concentrations remain high until the work output is returned to the control value. We subsequently realized that this type of biphasic response is seen after almost every intervention that produces an effect on P_i efflux in cardiac muscle, and rather similar results have been reported for nerve preparations by Straub *et al.* (1976).

Mild hypoxia (Fig. 6) also produced a transient increase in P_i efflux from working-heart pre-

Fig. 4. Continuous monitoring of $^{32}\text{P}_i$ uptake

The content of ^{32}P in a heart labelled in turn, for 1 min at a time, with (a) choline Ringer (46 mM- Na^+) and (b) sodium Ringer (146 mM- Na^+) was continuously monitored with a Geiger counter connected to a recorder. The specific radioactivity of $^{32}\text{P}_i$ was the same in each buffer (approx. $0.5 \mu\text{Ci}/\mu\text{mol}$) and the horizontal bars indicate the labelling periods. Perfusion with non-radioactive Krebs bicarbonate Ringer was continued between exposures to the radioactive solutions. The increase in $^{32}\text{P}_i$ uptake from one exposure to the next was determined from the increase in radioactivity measured from the slopes of the washout curves as shown above. The trace presented here is representative of many similar experiments.

Fig. 5. Effect of work load on $^{32}\text{P}_i$ efflux

Working preparations were loaded with $^{32}\text{P}_i$ at 13.3 kPa (100 mmHg) in Krebs bicarbonate Ringer/0.6 mM- P_i , as in the legend to Fig. 1. After rinsing, the hearts were equilibrated for 15 min at 6.7 kPa (50 mmHg) with buffer of the same P_i concentration. At the time indicated by the arrows the atrial-filling cannula was opened to increase the work demand, or closed to lower it.

parations, as might be partly expected from the rise in cellular P_i content under these conditions. Both respiratory and metabolic alkalosis initially decreased the rate of P_i loss (Fig. 7a), whereas acidosis

had the opposite effect (Fig. 7b). Once again it was possible to observe the return towards baseline values on prolonged stimulation. The effects of pH alterations produced by altering the CO_2 con-

Fig. 6. Effect of hypoxia on ${}^{32}\text{P}_i$ efflux

Working-heart preparations were used. Labelling and washout stages were done at 13.3 kPa (100 mmHg). The original solution [Krebs bicarbonate Ringer gassed with O_2/CO_2 (19:1)] was replaced with hypoxic medium gassed with $\text{O}_2/\text{N}_2\text{CO}_2$ (48:47:5).

Fig. 7. Effect of pH on ${}^{32}\text{P}_i$ efflux

The control buffer was 0.6 mM-P/Krebs bicarbonate Ringer. The concentration of NaCl was varied to compensate for changes in NaHCO_3 in the test solutions. (a) Effect of alkalosis; (b) effect of acidosis.

centration were seen more rapidly than those that occurred after a change in HCO_3^- concentration, which suggests that it is the intracellular pH that

determines efflux rate, since this is known to change more rapidly when CO_2 is varied rather than HCO_3^- . We also investigated the effect of alterations in the

anion composition of the perfusion buffer. P_i -free perfusion produced a slow change to a new lower steady-state rate of P_i loss, suggesting that there is no rapid P_i/P_i exchange across the cell membrane, and that the alterations observed probably stem from the gradual depletion of the cellular phosphate pool. A similar effect was observed (Fig. 8) when SO_4^{2-} and HCO_3^- were also replaced by Cl^- , confirming that HCO_3^- does not interact with the P_i carrier, in agreement with the results of the uptake experiments. (It should be noted that alterations from bicarbonate Ringer to artificially buffered media are difficult to perform without pacing problems and alterations in cardiac work output, so that a rather large series of hearts would be necessary to confirm that the bicarbonate effect is truly zero. It is certainly very small.)

Cl^- -free perfusion itself did not significantly affect the rate of P_i efflux, although it badly affected cardiac performance, and led to pronounced changes in coronary vascular resistance. Although a decline in P_i efflux was seen immediately after transitions to isethionate/Hepes Ringer (buffer C, Fig. 9), this is probably due to the decline in work output. Subsequently P_i efflux returned to, and even exceeded, control values on prolonged perfusion with Cl^- -free solutions, and this was associated with the development of heart failure, which in our hands is always characterized by high rates of P_i loss. It would appear that all these changes were associated with visible alterations in the metabolic status of the heart, and that P_i efflux can proceed at near-normal rates in the complete absence of permeant anions, arguing strongly against any role for P_i /anion exchange in the cardiac P_i -transport mechanism. This view was confirmed by further experiments in which even the permeant substrate anion (pyruvate)

Fig. 9. Effect of Cl^- -free perfusion on P_i efflux and cardiac performance

$^{32}P_i$ loading was done in the control Ringer with the hearts perfused in the Langendorff manner. The prelabelled hearts were then equilibrated in buffer B (Hepes Ringer) as working preparations. At the time indicated by the arrow (15 min) the perfusion solution was changed to buffer C (isethionate/Hepes) and perfusion with this buffer was continued until the heart failed. The atrial-filling rate [for 13.3 kPa (100 mmHg) aortic pressure in control buffer] was kept constant throughout the experiment. Sets of hearts were freeze-clamped after 15 min perfusion with buffer B ($n = 3$, control hearts), or after 15 min ($n = 4$) and after 40 ± 3 min ($n = 3$) perfusion with the Cl^- -free medium. The hearts from the last group were frozen when failure was imminent, as indicated by the position of the blocks on the Figure. The curve on $^{32}P_i$ efflux is representative of the general behaviour of these hearts. ■, P_i ; □, ATP; ▨, phosphocreatine.

Fig. 8. Effect of P_i and SO_4^{2-} -free perfusion on $^{32}P_i$ efflux

Perfusion was switched between Hepes Ringer and the test solution in which SO_4^{2-} and P_i had been replaced by Cl^- (buffer D, Table 1).

was replaced by 5 mM-glucose + 8 i.u. of insulin/litre, and when Cl^- was replaced by Mes buffer, which is presumably completely impermeant (buffer E, Table 1).

It is very difficult to vary the cation content of the perfusion buffer without consequential changes in membrane potential and work output, which in any case has already been shown to influence P_i efflux. We were obliged to perform all the remaining experiments with Langendorff preparations, since the hearts were unable to beat properly after alterations in Na^+ , K^+ and Ca^{2+} content of the perfusion buffers, and so they were unable to perfuse themselves as full working-heart preparations. As far as possible we sought to compare like with like and to provide a non-beating control in each case when the test solution led to disturbances in excitation-contraction coupling.

Replacement of 100 mM- Na^+ in the control buffer by choline (Fig. 10a) instantly produced a very large exit of $^{32}\text{P}_i$ from the heart, and this was followed by a slow decline to a new steady rate of loss, which was lower than the value originally observed in the control buffer. Returning to the control buffers (Krebs bicarbonate Ringer) produced a second smaller pulse of P_i efflux, followed by a slow return to the original basal efflux rate. In a second series of experiments, verapamil (0.25 mg/litre) was added to the perfusion medium in order to counteract the contracture that normally develops in low- Na^+ media. Verapamil partially inhibits Ca^{2+} entry into cardiac and vascular smooth muscle (Fleckenstein, 1971), and did not of itself produce any noticeable effect on P_i efflux (Fig. 10b), despite the large increase in coronary flow rate. Under these conditions low- Na^+ medium was still able to produce a large increase in P_i efflux, which could hardly be attributed to the change in work output, since the effect was in the wrong direction. It is possible, however, that the second peak in P_i efflux (Fig. 10a) on return to the control buffer was associated with the resumption of normal beating, since it was not observed in the experiment with verapamil (Fig. 10b) when contractions did not restart immediately after return to normal Na^+ concentrations. A choline titration (Fig. 10c) always elicited reversible increases in P_i efflux, with an initial overshoot apparent on a number of occasions.

Lowering the extracellular K^+ concentration leads eventually to an increase in the cellular Na^+ content (Ellis, 1977), although the details of this process are exceedingly complex, and involve changes in the membrane permeability to K^+ , Na^+ and Cl^- as well as a hyperpolarizing effect due to the electrogenic redistribution of K^+ , and a depolarizing effect due to the slowing of the electrogenic Na^+/K^+ pump (Katz, 1977). In our hands, low- K^+ Ringer invariably increased the rate of P_i efflux (Fig. 11a), but

high- K^+ Ringer failed to produce the expected reciprocal effect (Fig. 11b) unless very high concentrations were employed, leading to cardiac arrest. It is difficult to interpret the effects of K^+ alteration solely in terms of Na^+ -linked P_i efflux (though they do not exclude this possibility) especially when the effect of K^+ alterations on intracellular Na^+ in Purkinje fibres is known to be fairly modest (Ellis, 1977). No such objection can be made to the results with low- Na^+ Ringer, which was reported by Ellis (1977) to produce large and rapid alterations in the intracellular Na^+ content in Purkinje fibres, which would be entirely consistent with the observed effects on P_i efflux if there existed a Na^+ -linked P_i -export mechanism which under normal conditions suffers partial product inhibition by the high extracellular Na^+ concentration. On this basis the initial effect of Na^+ removal would be to accelerate transiently P_i efflux, as is in fact observed, followed by a slow decline to subnormal efflux rates. The alterations in membrane potential associated with changes in extracellular Na^+ were reported by Ellis (1977) to be small, although large decreases in membrane potential were associated with both high- and low- K^+ media. It seems to us likely that the cardiac P_i porter may well be sensitive to the membrane potential (as is indeed the case for the majority of ion channels studied to date), and that it operates faster when the membrane is depolarized. This would account both for the paradoxical effects of K^+ alterations, and for the massive accelerations in P_i efflux observed in severely failing hearts.

Perfusion with low- Ca^{2+} Ringer (0.6 mM) resulted in biphasic effects on P_i efflux, with an inverted response on return to normal buffer (Fig. 12). We were concerned that part of this response might be associated with an imbalance in cardiac energy supplies, since pyruvate dehydrogenase is a Ca^{2+} -dependent enzyme (Denton *et al.*, 1975), and the re-activation of pyruvate dehydrogenase after a change in work output in pyruvate-perfused hearts is known to be slow (Illingworth & Mullings, 1976a). It is apparent from Fig. 12 that this is unlikely to be the case, since an identical response was observed with both carbohydrates and lipid substrates for the heart. We feel that the effects of changes in Ca^{2+} concentration are secondary to the unavoidable changes in work output that take place at the same time, and that the same mechanisms were operative in the experiments shown in Figs. 5 and 12.

We performed a number of experiments with ion-transport inhibitors in an attempt to distinguish between the various anion-transport systems in cardiac muscle. Nitroprusside, a reagent known to inhibit Cl^- transport in vascular smooth muscle (Kreye *et al.*, 1977), did not affect P_i efflux at concentrations of either 25 or 130 μM (Fig. 13a), although the expected vasodilation was readily

Fig. 10. Effect of low Na^+ on $^{32}\text{P}_i$ efflux

(a) Langendorff prelabelled preparations were equilibrated in Krebs bicarbonate Ringer and switched to 100 mM-choline Ringer (46 mM- Na^+) at the times indicated by the arrows. The pump pressure was varied to maintain 13.3 kPa aortic pressure. PSP, Peak systolic pressure. (b) Perfusion with verapamil (0.25 mg/litre) was started 16 min before exposure to low Na^+ and maintained throughout perfusion with 46 mM- Na^+ Ringer, as indicated. (c) 100 mM-Choline Ringer and 146 mM- Na^+ Ringer were mixed to produce the final concentrations indicated. The heart was allowed to recover in 146 mM- Na^+ Ringer (Krebs bicarbonate Ringer) between exposures to choline solutions.

Fig. 11. Effect of K⁺ concentration on ³²P_i efflux

Langendorff prelabelled preparations were equilibrated in Krebs bicarbonate Ringer at 13.3 kPa, and the extracellular K⁺ concentration was varied as indicated. (a) Low K⁺; (b) high K⁺.

observed. Furosemide {Lasix; 5-(aminosulphonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid}, which is reported to inhibit Cl⁻ fluxes in frog heart (Brommundt *et al.*, 1978), barnacle muscle fibres (Ashley *et al.*, 1978), as well as P_i transport in erythrocytes and renal tissues (Brazy & Gunn, 1976), actually increased P_i efflux from the heart (see Fig. 13b). However, this potent diuretic is well known to cause gross alterations in cation permeability in kidney (Brazy & Gunn, 1976) and in Ehrlich ascites-tumour cells (Tupper, 1975), and it seems possible that its effects on cardiac P_i transport might be associated with changes in cation permeability in the heart. 4-Acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS), which

inhibits anion movements in erythrocytes without major alterations in cation fluxes (Cabantchik & Rothstein, 1972), and strongly inhibits Cl⁻ efflux in barnacle muscle fibres (Ashley *et al.*, 1978), only produced a small inhibition of cardiac P_i export (Fig. 13c). The same biphasic response was observed at drug concentrations of either 25 or 100 μM. Simultaneous application of furosemide and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) left P_i transport unaltered. These results confirm our earlier conclusion that there are fundamental differences between the cardiac P_i porter and the anion-transport system in erythrocytes, and strongly suggest that P_i and Cl⁻ are able to utilize separate carriers in rat heart. It is, however, very

Fig. 12. Effect of low Ca^{2+} perfusion on $^{32}\text{P}_i$ efflux

Langendorff prelabelled preparations were equilibrated in Krebs bicarbonate Ringer at 13.3 kPa and the extracellular Ca^{2+} concentration was varied from 2.5 to 0.6 mM, with carbohydrate or lipid substrate as indicated.

difficult to rule out the possibility that a small component of the total P_i efflux might utilize the electrogenic carrier mechanism which is presumably responsible for Cl^- movements in cardiac muscle.

The organomercurial thiol-group-blocking reagent mersalyl did produce an appreciable decrease in cardiac P_i losses (Fig. 13d), and this effect seemed to be additive to the inhibition produced by 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) (Fig. 13e). The problem with these inhibitors stemmed from their deleterious effects on cardiac performance, so that it was never entirely clear whether the recovery of P_i -efflux rates on continued perfusion resulted from the developing heart failure, or was yet another manifestation of the biphasic effects previously encountered in our experiments. We tested two other thiol-group blocking reagents in the hope of finding a better inhibitor, but 5,5'-dithiobisnitrobenzoic acid was without effect and *N*-ethylmaleimide completely poisoned the heart after a few seconds of exposure.

Biphasic effects on P_i efflux were observed after a number of disparate interventions in the experiments reported here, and our most recent unpublished observations suggest that this may be a rather general feature of cardiac P_i export. This raises obvious questions about the homogeneity of the cardiac intracellular P_i stores, and indeed leads us to question whether P_i transport was truly rate-limiting in our preparation. There are a number of observations that are relevant in this connection. (1) The total intracellular P_i pool is quite large (8–18 $\mu\text{mol/g}$ dry wt., depending on work load) when compared with the uptake rate (about 0.5 $\mu\text{mol/min}$

per g dry wt. at about 0.6 mM external P_i). (2) The intracellular P_i store is only gradually depleted by P_i -free perfusion (see Fig. 9), and there is no rapid P_i/P_i exchange.

The simplest explanation for these observations is that there is a single homogeneous intracellular P_i pool, with rate-limiting P_i transport across the plasmalemma. It is possible to devise more complex models involving multiple pools where transport across the plasma membrane is no longer the slowest step, but these models are subject to a number of severe constraints. In particular, any intracellular pool that communicates rapidly with the extracellular space must be very small, for otherwise its depletion would be apparent in P_i -free media. Multiple internal pools with rate-limiting transport across the plasmalemma remain a possibility, although our own unpublished work on the time course of $^{32}\text{P}_i$ incorporation into cardiac metabolites suggests other explanations for the non-equilibration of label throughout the cell.

It seems unlikely that changes in ATP turnover can directly explain any of the observations reported here. Even at moderate work outputs, cardiac pyruvate uptake (20 $\mu\text{mol/min}$ per g dry wt.; Illingworth *et al.*, 1975) corresponds to a complete turnover of the ATP γ -phosphate every 3 s, a result that also suggests the rapid equilibration of the mitochondrial and cytosolic pools of P_i in every one of our experiments.

In conclusion, it seems likely that P_i uptake and efflux in cardiac muscle are associated with the electroneutral co-transport of Na^+ ions, and it would not be surprising if the same protein catalyses both import and export. The approximate size of the

Fig. 13. Effect of anion-transport inhibitors on $^{32}\text{P}_i$ efflux

Hearts were perfused in the Langendorff manner with Krebs bicarbonate Ringer/0.6 mM P_i , at 13.3 kPa (100 mmHg) aortic pressure. Drugs were injected as concentrated solutions in the aortic flow with the help of a peristaltic pump to the final concentration and for the length of time indicated. (a) Effect of nitroprusside; (b) effect of furosemide (*very high end diastolic pressure); (c) effect of 4-acetamido-4'-isothiocyantostilbene-2,2'-disulphonic acid (SITS); (d) effect of mersalyl; (e) combined effect of 4-acetamido-4'-isothiocyantostilbene-2,2'-disulphonic acid (SITS) and mersalyl (*pacing problems).

transmembrane P_i gradient (approx. 5 mM total phosphate inside when the external concentration is 0.6 mM in our experiments) would be in approximate equilibrium with the oppositely directed Na^+ gradient [9.6 mM inside when the external Na^+ is 140 mM, from the data of Ellis (1977), based on an activity coefficient of 0.75] if the intracellular pH were slightly above 7 when the perfusion medium was at pH 7.4 and NaH_2PO_4 were the transported species. The acceleration of P_i efflux at low pH would be compatible with such a mechanism, although numerous other explanations can be devised for this effect. Since there was no sign of a rapid P_i/P_i exchange, and some indication for efflux inhibition by external Na^+ , one might suspect that the empty 'carrier' can cross the plasmalemma much faster in the unliganded state than when Na^+ and P_i are bound to it. Whether this is the only transport system for P_i or whether the cardiac cell is able to defend its internal P_i content with a second non-equilibrium carrier is a question that remains to be resolved at the present time.

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