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Correlations between cardiac protein synthesis rates, intracellular pH and the concentrations of creatine metabolites

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We have examined in detail the correlations between protein synthesis rates, intracellular pH (pH_i) and the concentrations of creatine metabolites in the rat heart perfused anterogradely *in vitro*. Using perfusion buffers ranging from pH 7.2 to 8.2 at 37 °C, we were able to manipulate pH_i from between 7.24 to 7.66, i.e. from the slightly acidotic to the alkalinotic as compared with the physiological values of pH_i (about pH 7.29). The dependence of pH_i on extracellular pH (pH_o) was linear, with the value of $\Delta pH_i/\Delta pH_o$ being 0.4–0.5. Protein synthesis rates were significantly stimulated when pH_i was increased above its physiological value, and they were strongly correlated with pH_i. They were also strongly correlated with phosphocreatine concentrations (and with creatine concentrations and phosphocreatine/creatine concentration ratios). Adenine nucleotide (ATP, ADP and AMP) concentrations and the ATP/ADP concentration ratio were not systematically altered by manipulating pH_i, and protein synthesis rates showed only a relatively weak dependence on these variables. Since creatine kinase catalyses a reaction that is close to equilibrium in the perfused heart, and since phosphorylation of creatine involves release of a proton, we argue that the changes in phosphocreatine and creatine concentrations are manifestations of alterations in pH_i. In this regard, we show that {log([phosphocreatine]/[creatine]) + log([ADP]/[ATP])} {the value of which gives [pH_i − log(mass action ratio)]} is positively correlated with pH_i, although the slope of the line is 0.7, as opposed to the ideal value of unity. We discuss three hypotheses to account for our observations: (i) protein synthesis rates are influenced directly by pH_i, (ii) pH_i affects the concentrations of creatine metabolites, which in turn affect protein synthesis rates, and (iii) pH_i affects the value of an unidentified co-variable, which in turn affects protein synthesis.

INTRODUCTION

In isolated cardiomyocytes, Purkinje fibres and the retrogradely perfused heart, pH_i can be conveniently manipulated by varying pH_o [1–4]. The dependence of pH_i on pH_o is relatively weak ($\Delta pH_i/\Delta pH_o$ = 0.2–0.4; [1–4]). We have recently shown that raising pH_o stimulates protein synthesis in the anterogradely perfused rat heart and in freshly prepared adult rat cardiomyocytes [5,6]. In perfused hearts, the maximal stimulation of protein synthesis by increased pH_o is as great as that by insulin [5]. We suggested that raising pH_o resulted in an increase in pH_i and that the increase in pH_i was responsible for the stimulation of protein synthesis. This suggestion was based on published data concerning the dependence of pH_i on pH_o in heart tissue preparations [1–4], and on our suggestion that the creatine kinase equilibrium could be used under these conditions (at least qualitatively) to assess pH_i because of the involvement of a proton in that reaction [5,7]. Thus, with respect to the latter, a rise in pH_o increases PCr concentrations, and increased protein synthesis rates are strongly correlated with PCr concentrations [5]. The possibility that protein synthesis was stimulated directly by PCr was not excluded. We were also aware that changes in pH_o and pH_i result in changes in the transport and intracellular concentrations of other ions, notably Na^+ and Ca^{2+}. These changes might also be relevant to the effects described. We have given some consideration to the possible physiological significance of our results [5]. We did not suggest that cardiac protein synthesis was directly modulated by pH_o *in vivo*, since pH_o remains relatively constant except in certain pathological states. We suggested that modulation of pH_i by hormones and growth factors (reviewed in [8]) might result, among other things, in a stimulation of protein synthesis. Further studies are needed in this area.

In our initial paper [5], we did not measure pH_i, but rather inferred a change using the creatine kinase reaction. We therefore decided to study the situation in greater detail. In particular, we wished to examine correlations between protein synthesis rate, pH_i and PCr, Cr and adenine nucleotide concentrations in the same heart. The results are reported here.

EXPERIMENTAL

Materials and animals

Chemicals and biochemicals were obtained from B.D.H., Dagenham, Essex, U.K., Sigma, Poole, Dorset, U.K., or Boehringer–Mannheim, Lewes, East Sussex, U.K. Hepes (Sigma) was cell-culture grade, and Tris (BDH) was Aristar grade. Radiochemicals and NCS were from Amersham International, Amersham, Bucks., U.K. All solutions were prepared with double-distilled deionized water. Male Sprague–Dawley rats (Harlan–Olac, Bicester, Oxon., U.K.) were kept in the animal house at 22 °C for at least 3 days before use. During this time, they had free access to food and water. Lighting was set for a 12 h-light/12 h-dark cycle, with the light phase beginning at 08:00 h.

General aspects of the anterograde heart perfusions

Hearts were perfused with modified Tyrode's solutions equilibrated with O_2. For the retrograde pre-perfusions and for the cardioplegic solutions, the buffers were prepared as described previously [5], except that the glucose concentration was 10 mM.

Abbreviations used: Cr, creatine; PCr, phosphocreatine; DMO, 5,5′-dimethyloxazolidine-2,4-dione; ECS, extracellular space; ICS, intracellular space; Γ, mass action ratio; the subscripts 'i' and 'o' refer to intracellular and extracellular values respectively.

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For the buffers for anterograde phase, 0.04 mM-phenylalanine and the remaining amino acids necessary to support protein synthesis (each at a concentration of 0.2 mM) were included before the pH was adjusted. The pH (at room temperature) and the buffering species used were: buffer A, pH 7.4, Hepes; buffer B, pH 7.8, Hepes; buffer C, pH 8.2, Hepes; buffer D, pH 8.7, Tris; buffer E, pH 9.1, Tris.

Hearts were removed from rats of 250–300 g body wt. and were perfused at 37 °C as described previously [9,10]. The pH of the buffer for the retrograde pre-perfusion was the same as that for the subsequent anterograde perfusions. After the completion of the cannulation procedure (during which the heart was perfused retrogradely at a pressure of 10 kPa), the preparation was switched to anterograde perfusion. The filling pressure was 0.5 kPa and the aortic pressure was 7.0 kPa. The final volume of the perfusate was about 100 ml. Zero time was taken as being 5 min (during which the heart was allowed to settle down) after the switch to anterograde perfusion. Throughout the perfusion period, pH_o was monitored with a pH-meter standardized at 37 °C.

The methodology used was complicated by the fact that hearts must be freeze-clamped in order to allow metabolite determination. This meant that a variable amount of extracellular fluid was trapped in the frozen heart. Thus pH_i could not be estimated by the simple methodology described previously [5]. Two sets of perfusions were therefore performed. In the first series, hearts were perfused for a total of 120 min with [4-^{3}H]-phenylalanine, [2-^{14}C]DMO and ^3H_2O (to measure the sum of the apparent ECS and the ICS). In the second series, the true ECS and ICS were determined by using ^3H_2O and [^{14}C]sucrose. From these measurements, all variables could be determined in the same heart.

Perfusions for 120 min in which protein synthesis, pH_i and metabolite concentrations were measured

Anterograde perfusions were allowed to equilibrate for 30 min. At 30 min, a flooding dose of 40 μmol of [4-^{3}H]phenylalanine (0.5 ml, 80 mM, sp. radioactivity 0.1 Ci/mol) was added to the perfusate. At 60 min, samples of perfusate were removed for measurement of the ^{3}H radioactivity (sample A). At 61 min, 0.5 ml of 100 mM-[2-^{14}C]DMO, pH 7.4 (sp. radioactivity 0.05 Ci/mol, pH adjusted with NaOH) was added. At 100 min, 0.1 ml, of ^3H_2O (sp. radioactivity 50 μCi/ml) was added. At 120 min, hearts were freeze-clamped with aluminium tongs cooled in liquid N_2 and were stored at −80 °C. Samples of perfusate were taken at this stage for determination of $^3H/^{14}C$ radioactivity (sample B). Radioactivity in samples A and B (1 ml) was measured with 10 ml of Fluoran HV (BDH) in an LKB 1219 scintillation counter standardized for $^3H/^{14}C$ dual-label counting in the same scintillant, according to the manufacturer's instructions.

The frozen heart tissue was extracted with 0.56 M-$HClO_4$ and processed for the determination of [4-^{3}H]phenylalanine incorporation into protein as described previously [11,12]. The total weight of protein present in the NaOH digest was measured by the biuret method [13] with BSA as a standard. A correction was made for the volume of the protein pellet that was digested in the NaOH, assuming a density of 1 ml/g. ^{3}H radioactivity was measured by using a toluene-based fluor. Quench curves were constructed by using NCS in the same fluor according to the manufacturer's instructions. The specific radioactivity of [4-^{3}H]phenylalanine in the perfusate was determined from the radioactivity and the phenylalanine concentration in sample A, the latter being determined by a tRNA-binding–isotope-dilution method [14,15]. The supernatant fractions from the initial $HClO_4$ extractions were neutralized [11,12], and PCr, Cr and the adenine nucleotides were determined by standard enzyme-coupled spectrophotometric techniques [16].

The sum of the apparent ECS and ICS in the freeze-clamped heart was determined by taking two samples (0.3 ml) of the neutralized supernatant fractions from the initial $HClO_4$ extractions. The $^3H/^{14}C$ radioactivity in one of these samples (sample C) was determined directly after the addition of 0.7 ml of water and 10 ml of Fluoran HV. The radioactivity in the other sample (sample D) was determined after evaporation to dryness by standing over P_2O_5 *in vacuo* for 48 h, the residue being dissolved in 1 ml of H_2O.

Measurement of the true ECS and ICS in hearts perfused for 120 min

Hearts were perfused anterogradely as described above in buffers A–D (two hearts at each pH). At 100 min, 0.1 ml of ^3H_2O (sp. radioactivity 50 μCi/ml) was added. At 110 min, 0.1 ml of [^{14}C]sucrose (10 μCi/ml, no additional unlabelled sucrose present) was added. At 120 min, the heart was removed from the cannulae, cut open, lightly blotted, weighed and stored at −80 °C. Samples (1 ml) of perfusate were taken for the determination of $^3H/^{14}C$ radioactivity as described above.

The heart was homogenized in a weighed tube in 5 ml of water with a Polytron homogenizer. A correction was made by weighing for incomplete recovery of homogenate in order to allow calculation of ECS and ICS in terms of ml/g wet wt. Trichloroacetic acid (0.25 ml of 100% w/v) was then added to the homogenate. After bench centrifugation at 4 °C, samples (1 ml) of the supernatant fraction were taken for $^3H/^{14}C$ dual-label counting in 10 ml of Fluoran HV as described above. Total protein in the pellet was measured as described above. Relative to heart wet weight, ECS was 0.389 ± 0.007 ml/g and ICS was 0.450 ± 0.006 ml/g (8 perfusions, means ± S.E.M.). Relative to protein, ECS was 2.362 ± 0.077 ml/g and ICS was 2.726 ± 0.044 ml/g (8 perfusions, mean ± S.E.M.). The latter is important, since it allows calculation of ICS in the first series of perfusions (see below). There was no evidence that changes in pH_o affected ICS or ECS.

Calculation of pH_i in hearts perfused for 120 min

The difference in ^{3}H radioactivity between samples C and D, and between samples A and B (i.e. ^3H_2O), allowed calculation of the apparent ECS + ICS relative to heart protein. The total protein content and the ICS/protein ratio (2.726 ml/g) gave the ICS in the frozen powder. Hence the apparent ECS in the frozen powder was obtained. The total [2-^{14}C]DMO radioactivity in the heart powder (i.e. that in the ECS and ICS) was calculated from samples C or D. The contribution of [2-^{14}C]DMO_o to this was calculated from the product of apparent ECS in the heart powder and the [2-^{14}C]DMO_o in sample B. Hence the total [2-^{14}C]DMO_i could be calculated by subtraction. Division of the total [2-^{14}C]DMO_i by the ICS in the frozen powder gave the concentration of [2-^{14}C]DMO_i. The [2-^{14}C]DMO_o concentration was known from sample B. Thus the DMO distribution ratio ($[DMO]_i/[DMO]_o$) could be calculated. Hence pH_i was calculated, by using the pH_o value at 120 min, from the formula:

$$pH_i = pK_a + \log\{([DMO]_i/[DMO]_o)(1 + 10^{pH_o - pK_a}) - 1\}$$

where pK_a is the value for DMO and was taken to be 6.28 [17].

Measurement of pH_i in hearts perfused for 30 min

In order to ensure that pH_i was stable over the period (30–120 min) during which protein synthesis was measured, a third series of perfusions was carried out. Hearts were perfused as described above. [2-^{14}C]DMO (0.5 mM, sp. radioactivity 0.05 Ci/mol) was present from the start of the anterograde

perfusion. To half of the perfusions, ^3H_2O (0.1 ml, sp. radioactivity 50 μCi/ml) was added after 10 min. To the remaining perfusions, 0.1 ml of [^{3}H]sucrose (50 μCi/ml, no additional unlabelled sucrose present) was added after 20 min. After 30 min, the heart was removed from the cannulae and processed as described above for the determination of ECS and ICS at 120 min and in ref. [5]. In these perfusions, ECS was 0.398 ± 0.008 ml/g wet wt. of heart and ECS + ICS was 0.839 ± 0.002 ml/g wet wt. of heart (10 perfusions, means ± S.E.M.). These values were not significantly different from those in the 120 min perfusions.

Other methods

Lactate in perfusates was assayed by a enzyme-coupled spectrophotometric assay [16]. Perfusate volume was estimated from the dilution of [2-^{14}C]DMO. Results are presented as means ± S.E.M. Data were analysed by using Graphpad software (ISI, San Diego, CA, U.S.A.) and a Microstat II statistics package (Ecosoft, Indianapolis, IN, U.S.A.). For second-order polynomial (with intermediate weighting) or linear regression analysis, statistical significance was taken as being established at $P < 0.0001$. For the two-tailed Student's t test other than when used in regression analysis, significance was taken as being established at $P < 0.05$.

RESULTS

Stability of pH_o in heart perfusions

Because of the temperature effects on the K_a of Hepes or Tris, the initial pH_o of perfusion buffers A–E was 0.2–0.4 pH unit lower at 37 °C than at room temperature (Fig. 1). Monitoring of pH_o revealed that there were decreases during the first 30 min of the perfusion period (Fig. 1), but that thereafter pH_o was relatively stable. The greater the initial pH_o, the greater was the initial fall in pH_o. There are several possible reasons for this finding. First, hearts perfused at higher pH_o values (buffers D and E) produce greater amounts of lactate over the initial 30 min perfusion period (Fig. 2). The reason for increased lactate release may be the increase in hydraulic work done, as demonstrated by the increased cardiac output seen at higher pH_o values [5]. Alternatively, the increased lactate release may be a manifestation of an effect of increased pH_i at the level of oxidative phosphorylation. Additional factors that may account for the early decrease in pH_o may include increased cardiac CO_2 production related to the increased hydraulic work done, the difference in buffering power between Hepes and Tris, the inherent variation in buffering power of the same buffer species at different pH values, and the greater tendency of the buffers to absorb atmospheric CO_2 at higher pH_o values.

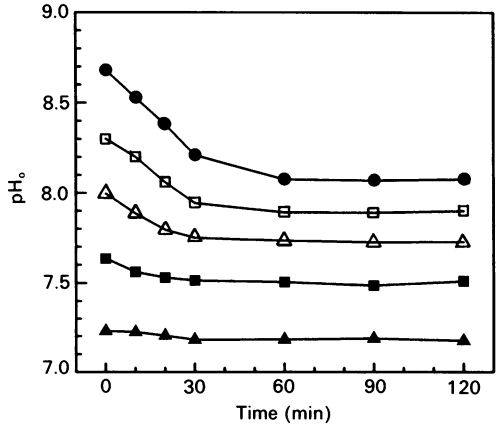

Fig. 1. Fall in pH_o with time of perfusion

Values are means from 4–10 separate perfusions. Hearts were perfused at 37 °C as described in the Experimental section with buffer A (▲, pH 7.4 at room temperature, Hepes), buffer B (■, pH 7.8 at room temperature, Hepes), buffer C (△, pH 8.2 at room temperature, Hepes), buffer D (□, pH 8.7 at room temperature, Tris), or buffer E (●, pH 9.1 at room temperature, Tris).

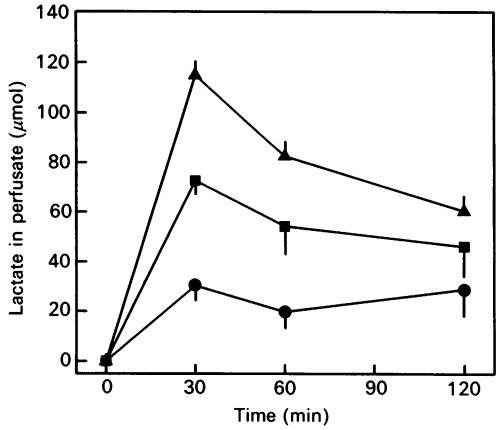

Fig. 2. Lactate production by hearts perfused with buffers of different pH_o values

Values are means from 4–6 separate observations at each time point. Hearts were perfused at 37 °C as described in the Experimental section with buffer A (●, pH 7.4 at room temperature, Hepes), buffer D (■, pH 8.7 at room temperature, Tris), or buffer E (▲, pH 9.1 at room temperature, Tris). Lactate production in buffers B or C was not significantly different from that in buffer A.

Dependence of pH_i on pH_o in perfused hearts

pH_i remained stable over the period (30–120 min) during which protein synthesis was measured (Table 1). pH_i was linearly correlated with pH_o (Fig. 3). There were no significant differences between the slopes of the lines or between the intercepts for the two groups of perfusions. $\Delta pH_i/\Delta pH_o$ was 0.4–0.5. We are not aware of any previous work using the anterogradely perfused heart in which the relationship between pH_o and steady-state pH_i has been explored. Our results (Fig. 3) agree reasonably well with data garnered from the literature, which is shown in Table 2. The

Table 1. pH_i values in hearts during perfusion with buffers of various pH_o values

Perfusion buffers were prepared and hearts were perfused as described in the Experimental section. The pH (at room temperature) and the buffering species used were as follows: buffer A, pH 7.4, Hepes: buffer B, pH 7.8, Hepes; buffer C, pH 8.2, Hepes; buffer D, pH 8.7, Tris; buffer E, pH 9.1, Tris. There were four separate observations at each pH in hearts perfused for 30 min and six separate observations for hearts perfused for 120 min.

Perfusion buffer	Perfused for 30 min		Perfused for 120 min	
	pH_o	pH_i	pH_o	pH_i
A	7.18 ± 0.01	7.24 ± 0.02	7.18 ± 0.03	7.24 ± 0.03
B	7.51 ± 0.01	7.36 ± 0.01	7.51 ± 0.03	7.38 ± 0.04
C	7.75 ± 0.01	7.44 ± 0.01	7.73 ± 0.04	7.41 ± 0.03
D	7.95 ± 0.02	7.56 ± 0.04	7.90 ± 0.03	7.60 ± 0.02
E	8.21 ± 0.01	7.68 ± 0.04	8.08 ± 0.02	7.66 ± 0.08

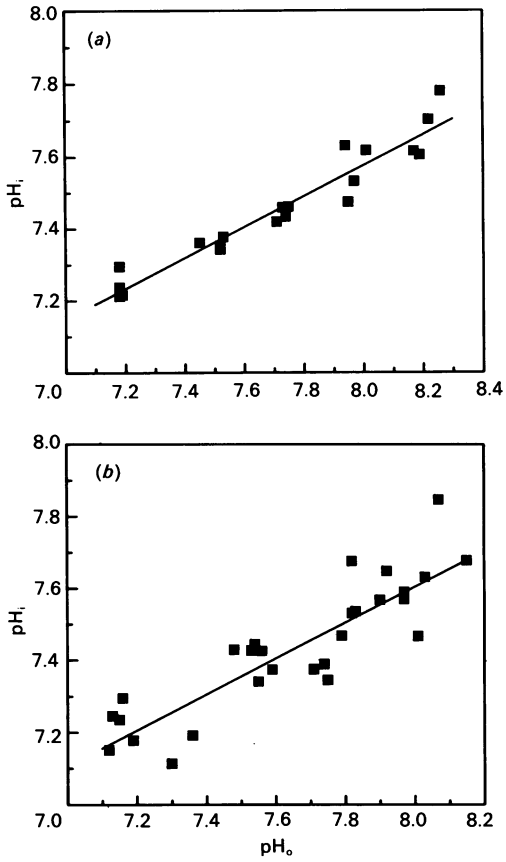

Fig. 3. Dependence of pH_i on pH_o

Linear regression lines are shown for hearts perfused for 30 min (*a*) or 120 min (*b*) with buffers A–E as described in the Experimental section. The pH_o values are those at the end of the perfusion period. For (*a*), the line is described by the equation $pH_i = 4.15 + 0.43\ pH_o$ ($r = 0.962$, $P = 1.39 \times 10^{-11}$, S.E.M. for intercept = 0.22, S.E.M. for slope = 0.03). For (*b*) $pH_i = 3.59 + 0.50\ pH_o$ ($r = 0.880$, $P = 6.88 \times 10^{-10}$, S.E.M. for intercept = 0.41, S.E.M. for slope = 0.05).

values of pH_i at a pH_o of 7.4 are about 7.0 for the epifluorescence measurements [3,4], but about 7.3 for our DMO measurements and the micro-electrode measurements by Deitmer & Ellis [1]. Some [18] (but not all; [2]) ^{31}P-n.m.r. measurements also indicate a pH_i of about 7.0. The reasons for these differences are not clear, but they are likely to be technical rather than real.

Dependence of protein synthesis rates on pH_i

By using second-order polynomial regression analysis, a significant correlation was demonstrated between protein synthesis rates and pH_i (Fig. 4 and Table 3). A pH_i value of about

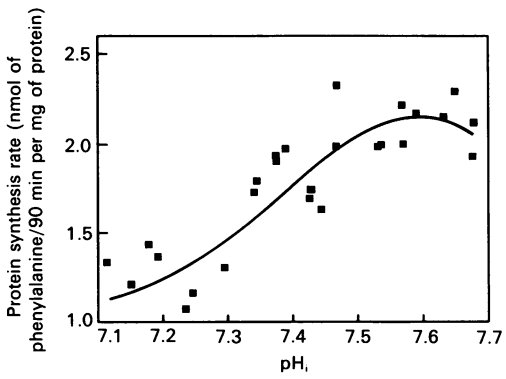

Fig. 4. Dependence of protein synthesis rates on pH_i

A second-order polynomial regression line is shown for hearts perfused for 120 min with buffers A–E as described in the Experimental section. Protein synthesis rates were measured over the 30–120 min perfusion period. The pH_i values are those calculated for the end of the perfusion period. The curve is described by the equation $y = 0.735 + 1150x - 469x^2 + 63.7x^3 - 2.88x^4$ ($r^2 = 0.802$, $P = 7.83 \times 10^{-9}$).

7.6 was optimal for protein synthesis. There was also a strong correlation between protein synthesis rates and pH_o (Table 3), probably the result of the dependence of pH_i on pH_o (Fig. 3). Since normal pH_o *in vivo* and *in vitro* is 7.4, the range of pH_o values studied here range from the acidotic to the alkalinotic. The implications in terms of the effects of intracellular acidosis or alkalosis on protein synthesis are discussed below. We also established that no ^3H from the ^3H_2O present in the perfusions was incorporated into protein, by processing the protein from hearts which were perfused for 120 min for the measurement of ICS and ECS.

Adenine nucleotide, PCr and Cr concentrations

There were no systematic changes in adenine nucleotide concentrations with increasing pH_o (Table 4). There were, however, significant increases in PCr concentrations and decreases in Cr concentrations, leading to significant increases in the PCr/Cr concentration ratio with increasing pH_o (Table 4). There were not significant ($P > 0.05$) changes in (PCr+Cr) between adjacent values of pH_o.

We have argued that the creatine kinase reaction can potentially assist in identifying a change in pH_i, since it involves the participation of a proton and is close to equilibrium in the perfused heart [5,7,19]. Thus, in the cytoplasm:

$$\Gamma = \frac{[PCr][ADP][H^+]}{[Cr][ATP]}$$

Hence: $pH_i - p\Gamma = \log([PCr]/[Cr]) + \log([ADP]/[ATP])$

Table 2. Dependence of pH_i on pH_o in cardiac tissue

The intercept and slope of the equation $pH_i = a + b(pH_o)$ were calculated from published data. Abbreviation: BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.

System	Method of pH_i measurement	Value of coefficient		Approx. range of pH_o studied	Reference
		a	*b*		
Sheep Purkinje fibres	pH-sensitive micro-electrode	5.59	0.23	5.4–8.4	[1]
Retrogradely perfused rat heart	N.m.r.	4.51	0.36	6.7–7.5	[2]
Neonatal rat isolated cardiomyocytes	BCECF epifluorescence	5.56	0.20	5–10	[3]
Adult rat isolated cardiomyocytes	BCECF epifluorescence	4.18	0.38	6–8	[4]

Table 3. Correlation of protein synthesis with pH_o and metabolite concentrations

The Table contains details of the correlations between protein synthesis rates (expressed in terms of nmol of phenylalanine incorporated/90 min per mg of protein) as the dependent variable (y axis) and pH_o or metabolite concentrations as independent variables (x axis) as examined by second-order polynomial regression analysis with intermediate weighting ($y = a+bx+cx^2+dx^3+ex^4$) for the 30 perfusions performed (six separate perfusions in buffers A–E), except for the correlation with pH_i, when there were 27 observations. All metabolite concentrations were expressed in terms of nmol/mg of protein. Details of the constitution and pH of the perfusion buffers are given in the Experimental section. The correlation was considered to be significant only if both of the following criteria were fulfilled: (i) $P < 0.0001$; (ii) the curve described by the regression equation displayed two or less points of inflexion within the limits of the measurements. The values of the five coefficients are not shown, but those for the correlations between protein synthesis rates and pH_i, PCr or Cr are given in the legends to Figs. 4, 6 and 7 respectively. For this form of regression analysis, the significance of the correlation between protein synthesis rate and the logarithmic value of the metabolite concentration is the same as for the untransformed value of the metabolite concentration. The correlation between protein synthesis rate and {log([PCr]/[Cr])+log([ADP]/[ATP])} is given, since {log([PCr]/[Cr])+log([ADP]/[ATP])} = $pH_i - pI'$.

Independent variable	r^2	P	Inflexions	Correlation significant?
pH_o	0.924	1.55×10^{-15}	1	Yes
pH_i	0.802	7.83×10^{-9}	1	Yes
PCr	0.909	1.59×10^{-14}	1	Yes
Cr	0.673	1.62×10^{-7}	2	Yes
PCr/Cr	0.877	7.06×10^{-13}	1	Yes
ATP	0.211	0.016	3	No
ADP	0.218	0.014	3	No
AMP	0.268	5.68×10^{-3}	3	No
ATP/ADP	0.416	2.81×10^{-4}	2	No
log([PCr]/[Cr])+log([ADP]/[ATP])	0.829	4.45×10^{-11}	1	Yes

Table 4. Adenine nucleotide, PCr and Cr concentrations in perfused hearts

Perfusion buffers were prepared and hearts were perfused for 120 min as described in the Experimental section. The pH (at room temperature) and the buffering species of the perfusion buffers were as follows: buffer A, pH 7.4, Hepes; buffer B, pH 7.8, Hepes; buffer C, pH 8.2, Hepes; buffer D, pH 8.7, Tris; buffer E, pH 9.1, Tris. There were six separate observations at each value of pH_o. Statistical significance was calculated by an unpaired Student's t test: $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ versus perfusions with buffer A.

	Metabolite concn. (nmol/mg of protein)								
Perfusion buffer	ATP	ADP	AMP	ATP+ADP +AMP	PCr	Cr	PCr+Cr	ATP/ADP	PCr/Cr
A	19.6±0.9	6.1±0.3	1.2±0.1	26.9±1.1	16.7±0.5	28.0±0.4	44.7±0.8	3.19±0.06	0.59±0.02
B	20.9±1.1	6.3±0.3	1.2±0.1	28.4±1.3	21.5±0.6^c	26.5±0.9	48.0±1.3	3.31±0.04	0.81±0.03^c
C	19.8±1.5	5.8±0.2	1.0±0.1	26.6±1.7	24.8±0.9^c	22.6±0.8^c	47.4±1.5	3.41±0.16	1.10±0.03^c
D	19.3±0.6	5.9±0.2	1.0±0.1	26.2±0.8	28.0±0.9^c	20.5±1.0^c	48.5±1.5	3.30±0.08	1.38±0.07^c
E	19.1±0.6	5.5±0.1^a	1.0±0.1	25.5±0.7	30.6±0.5^c	18.4±0.7^c	49.0±0.8^b	3.50±0.04^a	1.68±0.08^c

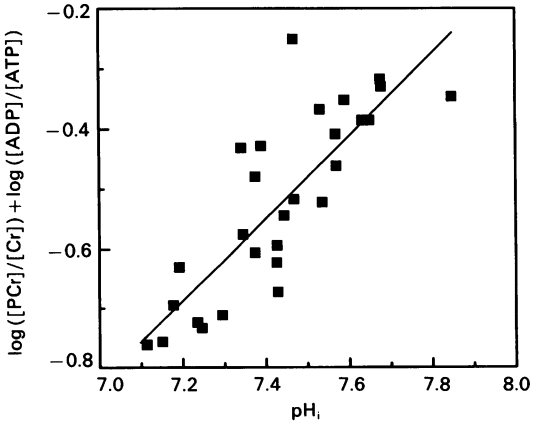

Fig. 5. Dependence of {log([PCr]/[Cr])+log([ADP]/[ATP])} on pH_i

A linear regression line is shown for hearts perfused for 120 min with buffers A–E as described in the Experimental section. Metabolite concentrations were measured and pH_i values were calculated at the end of the perfusion period. The line is described by the equation $y = 5.71+0.70x$ ($r = 0.823$, $P = 7.37\times10^{-8}$).

If this relationship holds, then a plot of {log([PCr]/[Cr])+log([ADP]/[ATP])} against pH_i should be linear, with a slope of unity. The experimentally determined relationship is shown in Fig. 5. The plot is indeed linear, with a slope of 0.70. Thus we conclude that the creatine kinase equilibrium may provide a qualitative indication of a change in pH_i, but that additional, more direct, evidence is necessary. Possible reasons for the divergence of the slope of the plot from unity will be discussed.

Correlations between protein synthesis rates and metabolite concentrations

The correlations as determined by second-order polynomial regression analysis are shown in Table 3. Protein synthesis rates were most significantly correlated with PCr concentrations (see also Fig. 6) and with the PCr/Cr concentration ratio. There was also a less significant correlation with Cr concentrations (see also Fig. 7). Only relatively weak correlations were seen between protein synthesis rates and adenine nucleotide concentrations. As expected, protein synthesis rates correlated significantly with {log([PCr]/[Cr])+log([ADP]/[ATP])} (Table 3). Since the ATP/ADP concentration ratio was relatively constant (Table 4),

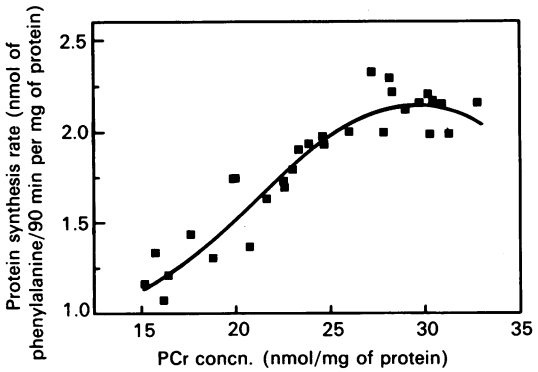

Fig. 6. Dependence of protein synthesis rates on PCr concentration

A second-order polynomial regression line is shown for hearts perfused for 120 min with buffers A–E as described in the Experimental section. Protein synthesis rates were measured over the 30–120 min perfusion period. The PCr concentrations are those measured at the end of the perfusion period. The curve is described by the equation $y = 8.941 - 1.420x + 0.089x^2 - 0.0022x^3 + (1.83 \times 10^{-5})x^4$ ($r^2 = 0.909$, $P = 1.59 \times 10^{-14}$).

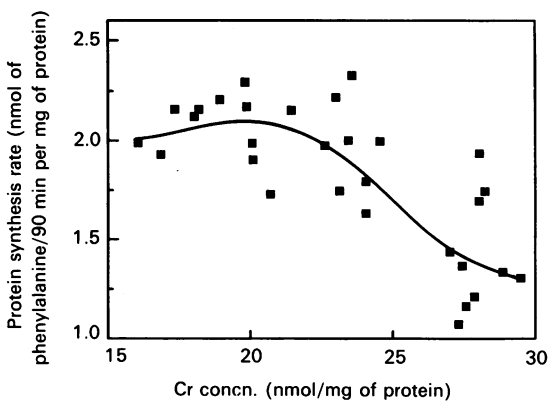

Fig. 7. Dependence of protein synthesis rates on Cr concentration

A second-order polynomial regression line is shown for hearts perfused for 120 min with buffers A–E as described in the Experimental section. Protein synthesis rates were measured over the 30–120 min perfusion period. The Cr concentrations are those measured at the end of the perfusion period. The curve is described by the equation $y = 40.871 - 7.815x + 0.577x^2 - 0.0184x^3 + (2.14 \times 10^{-4})x^4$ ($r^2 = 0.673$, $P = 1.62 \times 10^{-7}$).

the influence of the log([ADP]/[ATP]) term on the relationship was relatively small compared with that of the log([PCr]/[Cr]) term.

DISCUSSION

General points

The possible physiological significance of the stimulation of protein synthesis by increased pH_i is discussed in the following paper [20]. In our earlier experiments, we observed that increasing pH_o to greater than 8.0 stimulated protein synthesis rates and that the stimulation could be as great as that seen with insulin [5]. We also noted a correlation between protein synthesis rates and PCr concentrations. We suggested that increased pH_i might be responsible for both the increase in protein synthesis rates and the increase in PCr concentrations. These experiments were incomplete in at least two ways. First, pH_i was not measured directly, but was inferred from the work of others and from PCr concentrations. Secondly, we did not monitor pH_o throughout the perfusion period. Both of these shortcomings have now been corrected. The results reported here greatly extend our initial observations. In the current series of experiments, the rates of protein synthesis were measured in a manner slightly different from our normal method, where [U-^{14}C]phenylalanine is present at the start of the anterograde perfusion and the hearts are perfused for 90–120 min. The method was varied to allow pH_i to attain a steady state before protein synthesis measurements were initiated. On a technical point, although pH_o fell in the first 30 min of anterograde perfusion, we had to recirculate the perfusion buffer, because flow-through experiments would have been impossibly expensive.

Creatine kinase equilibrium and pH_i

The results in this paper confirm our previous suggestions [5] that the creatine kinase reaction may be useful under some circumstances as a qualitative indicator of changes in pH_i (Fig. 5). However, the slope of this plot is less than the theoretical value of unity. There are probably many reasons for this. Small variations in Γ might occur. Compartmentation of metabolites is another possibility. Creatine kinase catalyses a reaction that is primarily cytoplasmic. We measured total cellular concentrations of the creatine kinase substrates. Total ATP/ADP concentration ratios did not vary greatly with pH_o (Table 4). There is thus very little influence of the log(ADP/ATP) term on the slope of the plot of {log([PCr]/[Cr]) + log([ADP]/[ATP])} against pH_i. However, if increasing pH_i were to cause a disproportionate increase in cytoplasmic ADP/ATP concentration ratios, for which there is a compensatory decrease in the ADP/ATP concentration ratios in another compartment, the slope of the plot of {log([PCr]/[Cr]) + log(total [ADP]/total [ATP])} against pH_i that is observed would be decreased from its ideal value of unity. Equally, the DMO partition method measures the average cellular pH_i. If, on increasing pH_o, there were to be a disproportionately greater loss of intracellular protons from an unidentified compartment compared with the loss from the cytoplasm, the slope of the plot of {log([PCr]/[Cr]) + log([ADP]/[ATP])} against pH_i would again be decreased from its ideal value. Mg^{2+} also participate in the creatine kinase reaction [7], and this might also affect our findings. We are, however, relatively gratified that there are at least some grounds for using the creatine kinase reaction as a qualitative indicator of pH_i. We suggest that, when changes in the PCr/Cr concentration ratio are seen, it may be worthwhile measuring pH_i more directly.

Correlations between protein synthesis rates, pH_i and creatine metabolites

The results reported here show correlations between protein synthesis rates, pH_i and concentrations of PCr and Cr. There are at least three possible explanations. First, increased pH_i may stimulate protein synthesis directly. Secondly, increases in pH_i increase the PCr concentration and the PCr/Cr concentration ratio, and also decrease the Cr concentration. Protein synthesis rates may be modulated by the changes in the concentrations of creatine metabolites. Thirdly, the increases in pH_i may cause alterations in an unidentified co-variable, which modulates protein synthesis rates.

In cell-free translation systems, protein synthesis exhibits a very sharp pH optimum, although estimates of its value differ slightly in systems derived from the sea urchin [21,22]. It is thus feasible that small variations in pH_i may dramatically alter protein synthesis rates. Cell-free translation systems derived from the mammalian heart have not yet been developed. In the experiments reported here using perfusion buffers nominally free

of HCO_3^-, the physiological pH_i is 7.29 at a physiological pH_o of 7.4 (Fig. 3*b*). This value agrees well with the experimentally determined pH_i in HCO_3^--containing Krebs–Henseleit buffer at pH 7.4 [6,20]. If the pH optimum of cardiac protein synthesis is greater than 7.29, raising pH_i above 7.29 should stimulate protein synthesis and decreasing pH_i below 7.29 should inhibit it. This is what we observe (Fig. 4). The relationship in Fig. 4 also allows assessment of the likely effect of a small change in pH_i on the protein synthesis rate. At pH_i 7.29, the interpolated protein synthesis rate is 1.43 nmol of phenylalanine incorporated/90 min per mg of protein. If pH_i were to increase by 0.1 unit, the protein synthesis rate would increase by about 21%. It should be noted that, as well as protein synthesis being stimulated by an alkalinotic pH_i, it is also inhibited by an acidotic pH_i. In the heart, ischaemia causes intracellular acidosis [18] and inhibits protein synthesis [23]. The inhibition of protein synthesis may result from the intracellular acidosis, and this may limit the capacity of the cardiomyocyte to carry out diurnal replacement of protein and to repair.

Alternatively, the increase in protein synthesis rates induced by increases in pH_i may derive from alterations in the 'energy status' of the heart (Table 4). Protein synthesis rates are much more strongly correlated with the concentrations of creatine metabolites than with the concentrations of adenine nucleotides (Table 3). Translation requires hydrolysis of ATP and GTP. GTP/GDP concentration ratios are in equilibrium with those of ATP/ADP (which are in turn in equilibrium with $PCr \cdot H^+/Cr$ concentration ratios). It is thus possible that purine nucleotide concentrations could affect protein synthesis rates in some circumstances. This undoubtedly occurs when ATP and PCr are severely depleted. However, the weakness of the correlations (Table 3) probably precludes such a mechanism in the experiments reported here, unless compartmentation of adenine nucleotides is a factor. Alternatively, the protein synthesis machinery could itself be allosterically sensitive to the concentrations of creatine metabolites. This is a proposition which should be considered alongside the possibility that the action of pH_i on protein synthesis is direct.

Regulation of protein synthesis by other factors co-varying with pH_i

A rise in pH_o resulted in increased lactate production (Fig. 2). In buffers D or E, there was significant subsequent re-absorption (and presumably oxidation) of lactate after 30 min of perfusion. Lactate provided exogenously stimulates protein synthesis rates [24], increases PCr concentrations [11], but does not increase pH_i [20]. We therefore examined the correlation between protein synthesis rates and perfusate lactate concentration. There was no significant correlation ($P > 0.0001$) between protein synthesis and the lactate concentration in the perfusate at the 60 or 120 min time point. We did not attempt correlations at 30 min, since the two variables were measured in different hearts. However, there was a significant correlation ($P = 2.72 \times 10^{-6}$) between protein synthesis rates and lactate uptake over the 60–120 min perfusion period (results not shown). We do not think that endogenous production of lactate stimulates protein synthesis rates or increases PCr concentrations. In experiments in which lactate output was increased dramatically by perfusion of hearts under hypoxic conditions [11] or with the inhibitor of mitochondrial pyruvate transport, 2-cyanocinnamate (S. J. Fuller & P. H. Sugden, unpublished observations), there were no alterations in protein synthesis rates. In the perfusions with 2-cyanocinnamate, there were no alterations in pH_i or in the concentrations of adenine nucleotides or creatine metabolites. However, the possibility still remains that protein synthesis rates are related to rates of lactate uptake.

We do not know why increased pH_i increases lactate output. One possibility is that it increases cardiac hydraulic work [5], which may under our conditions of perfusion increase lactate output [10] and result in a greater dependence on anaerobic glycolysis for energy production. Alternatively, disturbances in the pH gradient across the mitochondrial membrane might lead to a decreased efficiency of oxidative phosphorylation and increased anaerobic glycolysis. We are not clear as to why the hearts perfused with buffers D and E in particular start to re-absorb lactate after 30 min (Fig. 2).

It is unlikely that the increase in hydraulic work induced by increased pH_i [5] increases protein synthesis rates, since it takes the form of a volume-overload as opposed to a pressure-overload. We have been unable to detect any effect of volume-overload on cardiac protein synthesis [25], although an effect of pressure overload is readily demonstrable [26] in the anterogradely perfused heart. In addition, an increase in pH_o stimulates protein synthesis in quiescent isolated cardiomyocytes [6].

We are also aware that there are complex interrelationships between pH_i, pH_o and the transport systems for and the intracellular activities of other ionic species such as Ca^{2+} or Na^+ [27–29]. It is thus possible that altered intracellular activities of these ions may regulate protein synthesis rather than pH_i. In general, it is thought that increases in pH_i favour decreased free Ca^{2+}_i and increased Na^+_i activities [1,3]. Changes in Ca^{2+}_i activities induced by alterations in pH_o and pH_i are probably mediated by altered membrane handling of Ca^{2+} and by release/uptake of Ca^{2+} from/to carboxy groups of intracellular protein-binding sites. Changes in Na^+_i activities are possibly mediated by Na^+/H^+ exchange. In many ways Ca^{2+}_i is an ideal messenger to link increased contractile activity to increased protein synthesis. An increase in pH_i should favour increased Ca^{2+} binding to proteins, and one such binding event could regulate protein synthesis. However, there is very little evidence that Ca^{2+}_i has any effect on cardiac protein synthesis [30]. With respect to Na^+, it has recently been suggested that the stretch-induced stimulation of protein synthesis in ferret papillary muscles is related to Na^+ influx [31]. In the same system, Na^+ ionophores also stimulated protein synthesis. Thus the importance of these ions cannot be discounted.

General conclusions and future directions

We have established highly significant correlations between protein synthesis rates and pH_i, and between protein synthesis rates and the concentrations of creatine metabolites (although we are aware that a correlation does not indicate a cause). We favour the explanation that protein synthesis is directly stimulated by an increase in pH_i, since it appears to us that there is a greater experimental justification of this interpretation, given the sharp pH optimum of protein synthesis in cell-free systems. We still need to provide unambiguous evidence to support this interpretation. We also need to examine more fully the effects of lactate on protein synthesis and to investigate which stage of translation is stimulated by increased pH_i.

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