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Determination of buffering capacity of rat myocardium during ischemia

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To determine the buffering capacity of ischemic rat myocardium, lactate production was altered by glycogen depletion prior to total global ischemia. Lactate production was monitored by ^{1}H-NMR spectroscopy in perfused rat hearts and determined by enzymatic assay of freeze-clamped tissue extracts. Intracellular pH was measured by ^{31}P-NMR spectroscopy. The relationship between total lactate produced and pH varied considerably, depending on the final pH reached. At pH > 6.4 this relationship is linear with a total buffering capacity (Δlactate/ΔpH) of 25 μmol H^+/g wet weight per pH unit. At lower pH values (pH < 6.4), the total buffering capacity increases progressively. Since ischemia is invariably accompanied by ATP and phosphocreatine (PCr) hydrolysis, the proton production/consumption during high-energy phosphate hydrolysis must be considered when evaluating the intrinsic buffering capacity of the myocaridum against proton loads produced by lactate production from glucose and glycogen. Schemes are presented which allow an estimation of the contribution of ATP and PCr hydrolysis and the buffering by the CO_2/HCO_3^- system during ischemia. At pH > 6.4, the majority (about 60%) of buffering is due to hydrolysis of adenosine triphosphate, phosphocreatine in the heart, and neutralization of sodium bicarbonate in the perfusate. At pH < 6.4 an increasing proportion of cardiac buffering is from intrinsic cardiac buffers, most likely from intracellular proteins. After correction for these contributions to the observed total cardiac buffering capacity, the intrinsic buffering capacity of the myocardium can be accounted for by a high capacity (170 μmol/g wet weight) but low pK_a (5.2) buffering system.

Introduction

The relative contributions of high-energy phosphate depletion, of intracellular acidosis, and the accumulation of intracellular lactate to cardiac injury in myocardial ischemia are not understood [1–3]. Phosphorous (^{31}P) nuclear magnetic resonance (NMR) spectroscopy is useful for investigating ischemia, since it monitors changes in high-energy phosphates and intracellular pH simultaneously and non-destructively [4]. The production of lactate during ischemia cannot be measured directly by ^{31}P-NMR, however.

Several investigators have suggested that there is a near-linear relationship between changes in proton concentration and intracellular pH in cardiac and non-cardiac tissue [5,6]. This relation-

Abbreviation: MDP, methylene diphosphonate.

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ship is commonly expressed as a buffering capacity, β, which is defined as the number of moles of strong acid required to decrease intracellular pH by 1 pH unit in one l of solution at a given pH [7].

$$\beta = \Delta[H^+]/\Delta pH \quad (1)$$

Since anaerobic glycolysis is the predominant source of H^+ production during myocardial ischemia, this equation can be expressed as:

$$\beta = \Delta[H^+]/\Delta pH \approx \Delta lactate/\Delta pH \quad (2)$$

Thus, with the knowledge of the buffering capacity of cardiac tissue, one can monitor changes in lactate production indirectly by measuring pH change with ^{31}P-NMR spectroscopy.

Widely ranging values for the buffering capacity of rat myocardium have been reported using different techniques and experimental conditions [5,6,8]. However, no one has reported a value of cardiac buffering capacity during myocardial ischemia where measurement of tissue pH and lactate concentration were made on the same tissue sample. We report the cardiac buffering capacity determined from a large number of hearts during myocardial ischemia. Intracellular pH, measured by ^{31}P-NMR spectroscopy, relative lactate production measured by ^{1}H-NMR spectroscopy, and absolute myocardial lactate concentrations measured by enzymatic assay were determined in each tissue sample. Furthermore, corrections for the contribution to proton production and utilization by ATP and PCr hydrolysis were also made using the fractional stoichiometry of each species and its various complexes with Mg^{2+} during hydrolysis [9–12]. These calculations are described in the Appendix.

Methods

Animals

Male Wistar rats weighing 280–320 g were used for all experiments. All animals were injected with 400 units of heparin intravenously under general anesthesia (diethyl ether) and killed by rapidly excising the heart. The hearts were then immediately immersed in ice-cold Krebs-Henseleit buffer [13].

Perfusion method

All hearts were perfused in the Langendorff mode by previously described methods [1]. Phosphate-free Krebs-Henseleit buffer containing glucose (11 mmol/l) was used with KH_2PO_4 replaced by an equivalent amount of KCl. The buffer was equilibrated with a mixture of 95% O_2/5% CO_2 and warmed to 37°C with a heat exchanger located just proximal to the probe. The hearts were perfused with an aortic pressure of 70 cm of water which resulted in coronary flow rates of 10 to 15 ml/min and heart rates of 250–300 beats/min. The temperature in the perfusion chamber was maintained at 37°C by flowing thermostatically heated air around the perfusion chamber while the probe was within the bore of the magnet.

The hearts were subjected to periods of substrate-free perfusion ranging from 0 to 90 min prior to the onset of total global ischemia. Ischemia was induced by stopping the flow of buffer to the heart.

Prior to total global ischemia, baseline intracellular pH was determined by ^{31}P-NMR spectroscopy. During total global ischemia, myocardial lactate production was determined by serial ^{1}H-NMR spectroscopy acquired with a time resolution of 1 min. When lactate concentration was observed to be constant, intracellular pH was again measured by ^{31}P-NMR spectroscopy. This was generally after 20 min of total global ischemia (see below). The hearts were then freeze-clamped between aluminum plates which had been cooled in liquid N_2. The hearts were extracted with perchloric acid and assayed for lactate as described below. The perfusate surrounding the heart (approx. 11 ml) was also removed and assayed for lactate.

NMR methods

Probe design. The probe for perfused hearts was constructed with single-turn Helmholtz coil that was tuned to both ^{31}P and ^{1}H using the design of Hoult [14]. This permitted the sequential acquisition of both ^{31}P and ^{1}H spectra on the same heart. The hearts were perfused with a vertical orientation in the coil. Standards of methylene diphosphonate (MDP) and tetradeutero sodium propionate in ^2H_2O were placed in individual capillary tubes within the annulus of the sample tube.

pH determination. ^{31}P spectra were acquired at 73.84 MHz as described previously [1]. Spectra were accumulated over a period of 6 min using a 70° pulse angle and an interpulse delay of 1 s. Data was collected in 4K data points and exponentially multiplied to give a line broadening of 15.0 Hz prior to Fourier transformation. The intracellular pH was determined from the position of the inorganic phosphate (P_i) peak relative to the peaks of either phosphocreatine or the MDP standard [5,1] (Fig. 1).

Lactate measurements by NMR. Proton spectra were acquired at 182.4 MHz. Proton spectra were acquired using a $1\bar{3}3\bar{1}$-τ-$2\bar{6}6\bar{2}$-τ-Acquire spin-echo pulse sequence in order to suppress the water resonance [15,16]. The delay, τ, was usually set at 68 ms. The excitation band was centered at the resonance frequency of the lactate methyl group which is 3.4 ppm upfield from the water resonance. Sequential 1 min spectra were acquired during the period of total global ischemia (Fig. 2). A small signal from the CH_2 of triacylglycerol was noted in the same region as the lactate peak in each heart [17]. However, this did not interfere with our observation of increases in lactate peak height during ischemia. An observed 'plateau' in myocardial lactate production was used to define the endpoint in each experiment after which the pH was determined by ^{31}P-NMR spectroscopy and the heart was freeze-clamped.

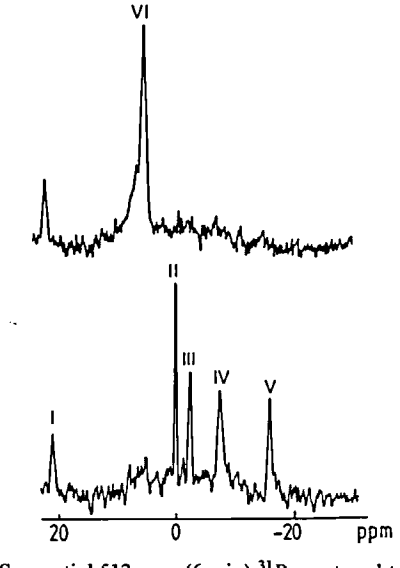

Fig. 1. Sequential 512 scan (6 min) ^{31}P spectra obtained during normoxic perfusion (lower panel) and after 25 min of total global ischemia (top panel). The final pH was measured from the chemical shift of P_i relative to MDP. Resonance identifications: I, MDP; II, PCr; III, γ-ATP; IV, α-ATP; V, β-ATP; VI, P_i.

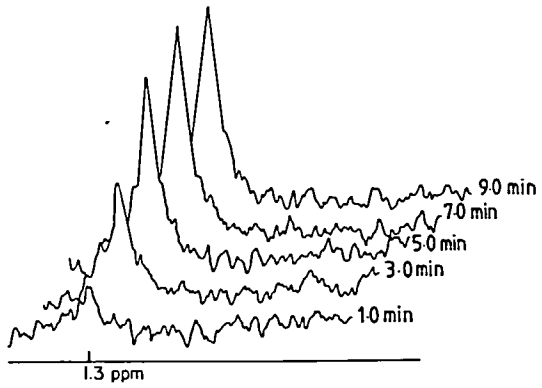

Fig. 2. Sequential 64 scan (2.0 min) $1\bar{3}3\bar{1}$-τ-$2\bar{6}6\bar{2}$-τ-spin echo spectra showing the lactate methyl resonance, located 1.3 ppm downfield from the tetradeutero sodium propionate standard, during a period of total global ischemia. The times shown are the midpoint times of the spectra following onset of ischemia at $t = 0$.

Tissue lactate assays

Assays of myocardial lactate were made on neutralized perchloric acid extracts of the freeze-clamped hearts [18]. Lactate in the freeze-clamped tissue and in the perfusate surrounding the heart in the probe chamber is expressed as μmol lactate/g wet wt. of heart tissue. The wet/dry wt. ratio of the ischemic heart at the end of the experimental protocol was 5.58 ± 0.50 (S.D.) ($n = 19$). Total lactate produced during ischemia was the sum of the heart lactate and the chamber perfusate lactate.

Tissue adenine nucleotide, nucleoside and purine assays

Assays of myocardial adenine nucleotides, nucleosides and purines were performed on neutralized perchloric acid extracts of four hearts that were freeze-clamped after 30 min of total global ischemia. Analysis of adenine nucleosides and purines was performed by high performance liquid chromatography (HPLC) [19]. Analysis of adenine nucleotides was performed by enzymatic assay [18] as well as by HPLC [20].

Results

Relationship of lactate production to pH

The amount of myocardial lactate produced upon total global ischemia was varied by subjecting the hearts to variable periods (0–100 min) of substrate-free perfusion prior to ischemia. This depletes cardiac glycogen to a variable extent [1] resulting in a variable extent of lactate production. The relationship between intracellular pH and total lactate produced during ischemia is shown in Fig. 3. Tissue lactate concentration was assumed to be 0.5 μmol/g wet wt. prior to the onset of ischemia [21]. Substrate-free perfusion for the 0–100 min periods employed did not result in significant accumulation of myocardial lactate as evidenced by the absence of significant changes in the lactate region of the ^{1}H-NMR spectrum during the period of substrate-free perfusion. The pH was determined from ^{31}P spectra obtained just prior to freeze-clamping the heart 25–30 min after the onset of ischemia. Both the pH and lactate concentration were observed to be constant at this time.

Before the onset of ischemia, lactate in the perfusion chamber was less than 0.1 mmol/l. Upon cessation of flow, variable amounts of lactate were observed in the medium surrounding the heart which were equivalent to 15–50% of the total lactate formed during ischemia.

As with all buffer systems, the observed buffering capacity will depend on the total concentration of buffer species and the pK_a of the buffer in relation to the observed pH. At pH > 6.4, the relationship between total lactate production and ΔpH is approximately linear with a total buffering capacity (Δlactate/ΔpH) of 25 μmol H^+/g wet wt. per pH unit. At final pH values between 6.4 and 6.1, the total buffering capacity is 45 μmol H^+/g wet wt. per pH unit. At a pH < 6.1 there is considerably more scatter in the relationship between Δlactate/ΔpH. However, the overall trend is that greater amounts of lactate are required to lower the pH by 1 unit with a total buffering capacity of 55–65 μmol H^+/g wet wt. per pH unit in this region. These observed relationships reflect the sum of protons produced or consumed by the hydrolysis of ATP and PCr, buffering by P_i and bicarbonate (HCO_3^-), and intrinsic cardiac buffering which includes buffering by intracellular proteins and net proton pumping from the intracellular space to extracellular compartments.

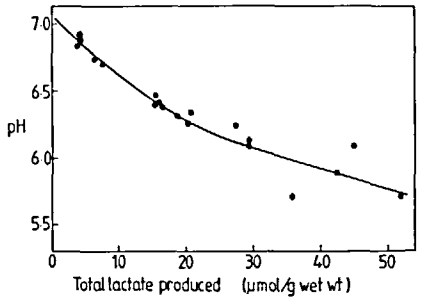

Fig. 3. The relationship between intracellular pH and total lactate produced during ischemia. Intracellular pH was determined by ^{31}P-NMR spectroscopy prior to freeze-clamping the hart. Total lactate production was determined by enzymatic assay. Each point represents a single measurement of one heart.

Products of ATP hydrolysis

Of the ATP available before the onset of ischemia, analysis of total adenine nucleotide, nucleoside and purines in the heart after 30 min of ischemia showed that more than 87% of the total ATP was hydrolyzed, giving a mixture of adenosine monophosphate (AMP) (50%), inosine monophosphate (IMP) (less than 2%), adenosine (15%), inosine (16%), hypoxanthine (4%), xanthine (14%) and uric acid (less than 1%). Myocardial adenosine diphosphate (ADP) content was constant before and after total ischemia (0.64 ± 0.11 μmol/g wet wt.).

Proton consumption / contribution by ATP and PCr hydrolysis and by P_i and HCO_3^- neutralization

The number of protons produced by the hydrolysis of ATP depends not only on the pH but also on the free magnesium concentration [Mg^{2+}]. The free [Mg^{2+}] also varies with pH so that calculation of the number of protons produced or consumed during ATP hydrolysis requires calculation of the concentration of Mg^{2+} complexes as well as the concentration of various protonation states of ATP and P_i. The net hydrolysis reaction can be represented by the overall equation.

$$ATP \cdot Mg^{n} + 3H_2O \longrightarrow AdoH + 3P_i^{m} + a\,Mg^{2+} + b\,H^{+} \quad (3)$$

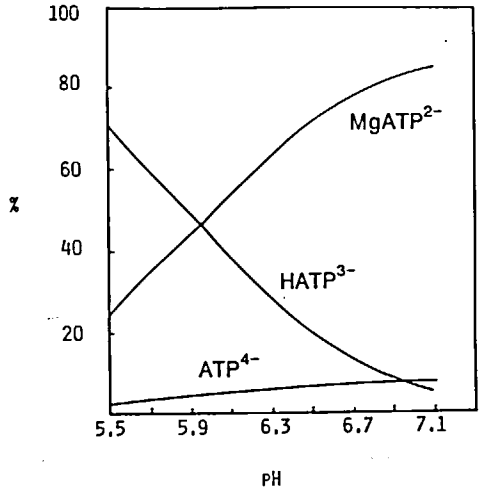

Fig. 4. (A) The percentage of ATP that exists as $MgATP^{2-}$, $HATP^{3-}$ and ATP^{4-}, versus pH assuming a free $[Mg^{2+}]$ of 1 mmol/l. Calculations are described in the Appendix.

where $n = 3m + 2a + b$ (charge conservation) and AdoH represents the adenosine produced from complete hydrolysis of ATP (H is necessary in this term to balance the equation with respect to protons). The coefficients n and m may be fractional and denote the average net charge of the species at a given pH. A complete discussion of the corrections for ATP and PCr hydrolysis is given in the Appendix.

Fig. 4 shows the percentage of ATP that exists as ATP-Mg^{2-} complex, H-ATP^{3-}, and ATP^{4-} over a range of pH values and a physiological Mg^{2+} concentration (0.001 M) [22]. As the pH falls, there is a decline in the percentage of the ATP-Mg^{2-} and ATP^{4-} species and an increase in the H-ATP^{3-} species. The H-ATP · Mg^{1-} species is present in a low concentration over this pH range (less than 1%). Very little P_i exists as P_i-Mg complex over a pH range of 5.5–7.1 (less than 5%). The above data were derived from dissociation constants given by Alberty [9]. Phosphocreatine does not complex significantly with Mg^{2+} in vivo over this pH range [12].

Fig. 5A shows the relative proton consumption/release due to ATP and PCr hydrolysis over a range of pH values. The calculations were made using the fractional stoichiometry shown in Fig. 4 and assuming that the initial concentrations of total ATP (all species) and PCr were 4.8 and 6.8 μmol/g wet wt. as determined in Refs. 21 and 23. Note that although PCr hydrolysis consumes protons throughout this pH range, ATP hydrolysis to adenosine and $3\,P_i$ releases protons at $pH > 6.5$ and consumes protons at lower pH values.

Proton consumption due to neutralization by P_i and HCO_3^-

Fig. 5B shows the proton consumption from neutralization of P_i that is present in myocardium prior to ATP and PCr hydrolysis. These calculations were made using the partial stoichiometry of the P_i species over this pH range calculated from the dissociation constants given by Alberty [9] and assuming a total P_i concentration of 1.5 μmol/g wet wt. [24,25]. This figure also shows the proton consumption due to neutralization by HCO_3^- present in the perfusate (24 mmol/l). These calculations were made assuming that CO_2 and HCO_3^- equilibrate across cell membranes.

Fig. 5C shows the net proton consumption/release due to both ATP and PCr hydrolysis and neutralization by P_i and HCO_3^-. Note that there is net proton consumption at $pH < 7.05$, which will produce a net buffering effect during ischemia.

Intrinsic cardiac buffering capacity

The concentration of ATP, PCr and P_i prior to ischemia in hearts subjected to substrate-free perfusion was determined by comparing the peak heights in the ^{31}P spectrum immediately before ischemia to those obtained before substrate-free perfusion. Baseline levels of ATP, PCr and P_i were assumed to be 4.8, 6.8 and 1.5 μmol/g wet wt., respectively, as determined by others (see above). Peak heights were measured relative to a standard of MDP.

Fig. 6 shows the relationship between net protons buffered versus pH after corrections for both ATP and PCr hydrolysis and P_i and HCO_3^- neutralization have been made. These corrections account for the actual reaction products observed on ATP hydrolysis according to Eqn. A-8, in the Appendix. This curve represents the actual intrinsic buffering capacity of the myocardium. Thus, intrinsic cardiac buffering capacity is defined as buffering by cardiac buffers other than ATP/PCr hydrolysis or neutralization of P_i or HCO_3^-. Thus defined, intrinsic buffering includes buffering by

proteins as well as activity of any proton pumps during ischemia.

Inspection of Fig. 6 shows that the effective buffering by intrinsic cardiac buffers increases with decreasing pH, suggesting a low effective pK_a for the residual cardiac buffers. Note that at pH > 6.4, this curve is approximately linear with an intrinsic buffering capacity ranging between 7 and 13 μmol H^+/g wet wt. per pH unit. However, at pH < 6.4, there is a progressive increase in intrinsic intracellular buffering capacity. This curve, drawn using a least-squares fit, indicates a total buffer concentration of 150 μmol/g wet wt. and an effective pK_a of 5.2. Also, it should be noted that the intrinsic buffering capacity was determined using the initial portion of the curve in Fig. 6 (pH $\geq$ 6.1). There is considerable scatter in the data at pH < 6.1 because of inaccuracy in measuring intracellular pH using ^{31}P-NMR spectroscopy in this pH range.

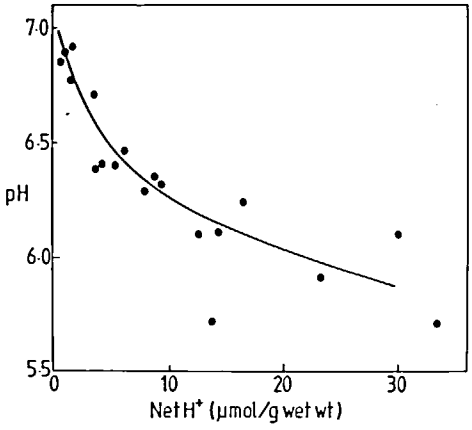

Fig. 6. The net protons buffered by cardiac buffers other than ATP, PCr, P_i and HCO_3^- versus pH. This curve represents the 'intrinsic' buffering capacity of cardiac tissue. Correction for the buffering capacity due to ATP and PCr hydrolysis and by the bicarbonate and phosphate buffers is described in the text. Each point represents a single measurement on one heart.

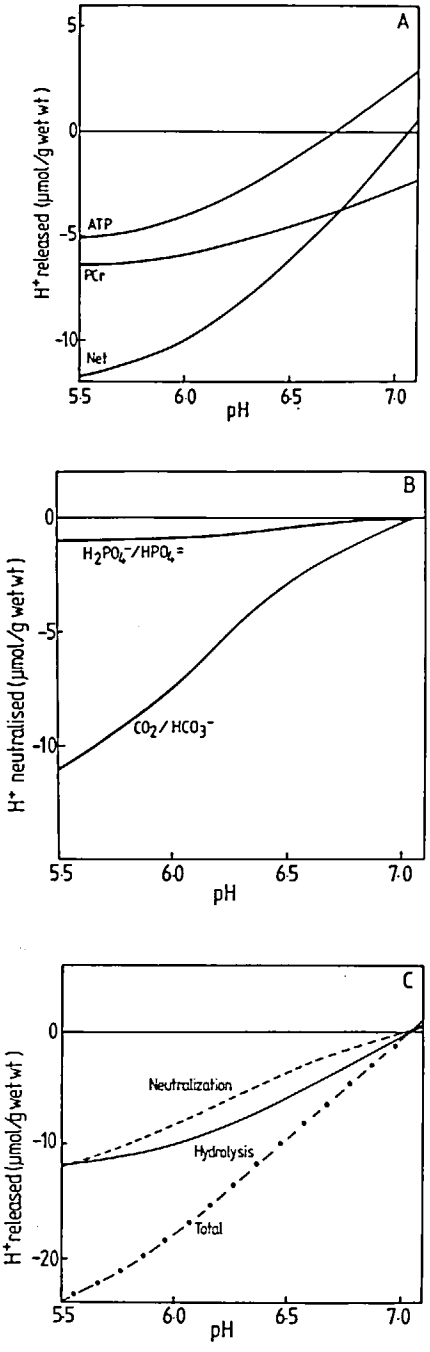

Fig. 5. (A) The proton release/consumption due to complete hydrolysis of ATP and PCr versus pH. These calculations were made assuming initial ATP and PCr concentrations of 4.8 and 6.8 μmol/g wet wt. as described in the appendix. (B) The proton consumption due to neutralization represents buffering by P_i that is present prior to any ATP/PCr hydrolysis and by HCO_3^- that is present in the perfusate. These calculations assumed an initial P_i concentration of 1.5 μmol/g wet wt. and an HCO_3^- concentration of 24 mmol/l as described in the Appendix. (C) The net proton consumption/release due to net hydrolysis of ATP/PCr, net neutralization by P_i/HCO_3^-, and both hydrolysis and neutralization. Negative values imply proton consumption (buffering) by the designated process.

TABLE I

CONTRIBUTION OF VARIOUS SYSTEMS TO CARDIAC BUFFERING DURING ISCHEMIA AT VARIOUS FINAL pH VALUES

Values are the average ± S.D. for hearts of comparable final pH value.

	Final pH	Total lactate production during ischemia (μmol H^+/ g wet wt.)	Substrate-free perfusion (min)	Initial concn. [a] (μmol/g wet wt.)			Protons buffered by: [b] (μmol/g wet wt.)			
				ATP	PCr	P_i	ATP/PCr /P_i [c]	HCO_3^- /CO_2	intrinsic cardiac buffers	total protons buffered
$n=3$	6.9±0	3.8±0.2	88.0± 3.5	1.9±0.4	2.4±0.3	15.0±1.3	1.4 (50)	0.6 (21)	0.8 (29)	2.8
$n=4$	6.4±0	17.0±3.0	48.8±14.3	4.6±0.3	4.0±1.0	6.6±3.6	6.7 (39)	3.7 (22)	6.8 (39)	17.2
$n=3$	6.1±0.1	34.0±9.0	0	4.7±0.5	5.5±1.2	1.5±0	8.6 (29)	6.3 (21)	15.0 (50)	29.9

[a] These values represent initial concentrations after substrate-free perfusion but prior to myocardial ischemia. The values were calculated from peak heights of ATP and PCr relative to the MDP standard on ^{31}P spectra and assuming initial concentrations of ATP, PCr and P_i as 4.8, 6.8 and 1.5 μmol/g wet wt. prior to substrate-free perfusion.

[b] These values were calculated from data of Figs. 5A and B and 6. The values in parentheses represent the percentage of total protons produced which are buffered by the individual groups.

[c] These values include protons buffered by the hydrolysis of ATP and PCr, as well as endogenous phosphate initially present prior to ischemia.

Table I tabulates the relative contribution of various systems to cardiac buffering at various pH values. Note that shorter periods of substrate-free perfusion give rise to increased lactate production and a lower final pH following ischemia. Also note that the concentrations of ATP and PCr are reduced and the P_i concentration increased with longer periods of substrate-free perfusion (prior to ischemia), reflecting an imbalance between ATP/PCr production and utilization when glucose is absent. At a pH of 6.9, hydrolysis of ATP/PCr and neutralization of bicarbonate account for 50% and 21% of protons buffered, respectively. At this pH the intrinsic cardiac buffering capacity accounts for only 29% of protons buffered. At a pH of 6.1, the total number of protons buffered has increased approx. 10-fold. However, at this lower pH, the percentage of protons buffered by intrinsic cardiac buffers has increased to 50% of the total, with those buffered by hydrolysis of ATP and PCr and by neutralization by bicarbonate and CO_2 accounting for the rest.

Discussion

There have been three previous studies that have reported values of buffering capacity for cardiac tissue. Two studies report values obtained from normoxic tissue ranging from 31 to 33 μmol H^+/g wet wt. per pH unit [5,6]. A third study reports the buffering capacity in ischemic myocardium in a small number of hearts [8].

In the first study, a buffering capacity ranging from 31 to 33 μmol H^+/g wet wt. per pH unit was determined by measuring the pH changes in homogenized rat ventricle at various CO_2 concentrations in 7–11 samples [5]. This study used relatively invasive techniques that might alter the metabolic integrity of the tissue. Furthermore, since it did not evaluate buffering capacity during ischemia, no correction for ATP or PCr hydrolysis was made, although such changes undoubtedly occurred upon tissue homogenization.

In the second study, the buffering capacity was determined in strips of rat left ventricle by measuring intracellular pH changes in response to changes in external CO_2 concentration by pH-sensitive micro-electrodes [6]. A buffering capacity of 33 μmol H^+/g wet wt. per pH unit was determined from four tissue samples. Conversion from units of mequiv. H^+/pH unit per l was made assuming that the cytosolic water volume is 0.43 ml H_2O/g wet wt. [21] and that the units were originally expressed in terms of cytosolic water volume. The buffering capacity was based on the

minimal transient pH observed on rapidly changing pCO_2 in the perfusate. After a short decrease in intracellular pH, the pH increased to a new higher value, presumably due to H^+ export and/or HCO_3^- import from the extracellular space. Again, this study did not account for ATP or PCr hydrolysis that might have occurred during preparation of the tissue samples or insertion of the microelectrodes.

A third study by Matthews reports values of cardiac buffering capacity ranging from 10 to 21 μmol H^+/g wet wt. per pH unit during myocardial ischemia in five rat hearts [8]. Changes in pH were measured by ^{31}P-NMR spectroscopy. Tissue lactate concentrations were determined by biochemical assay on a separate group of animals. The contribution of ATP and PCr hydrolysis was addressed, but no correction for the fractional stoichiometry of the various ATP species at varying pH was made. Furthermore, lactate that diffused into the perfusate surrounding the heart was not measured. We found that this ranges from 15–50% of the total lactate produced and failure to consider this results in significant underestimation of the cardiac buffering capacity.

The present study demonstrates that the total buffering capacity ranges considerably with pH during myocardial ischemia. At pH values above 6.4, the total buffering capacity is approx. 25 μmol H^+/g wet wt. per pH unit, whereas at pH values below 6.4, the total buffering capacity ranges from 45 to 65 μmol H^+/g wet wt. per pH unit (see Fig. 3). These values represent intrinsic cardiac buffering that occurs as a result of ATP and PCr hydrolysis, cellular inorganic phosphate and from bicarbonate in the perfusate.

This study demonstrates that the intrinsic buffering capacity also varies with pH. At pH values above 6.4, the intrinsic cardiac buffering capacity ranges from 7.0 to 13 μmol H^+/g wet wt. per pH unit (see Fig. 6). In this range, the intracellular pH is more dependent on ATP and PCr hydrolysis and proton buffering from HCO_3^- in the perfusate than on the physico-chemical buffering of cardiac tissue per se. At lower final pH values (below 6.4) the intrinsic buffering capacity accounts for an increasing fraction of the total protons buffered. For example, at a final pH of 6.1, the intrinsic cardiac buffering accounts for 55% of all protons consumed, whereas ATP and PCr hydrolysis and bicarbonate neutralization each account for 22% and 18% of protons consumed (see Table. I). After correcting for the contributions of ATP/PCr hydrolysis and neutralization of bicarbonate and P_i to total cardiac buffering, the 'intrinsic' buffering capacity of myocardium can be accounted for by a high capacity (170 μmol/g wet wt.), but low pK_a system. The intrinsic cardiac buffering capacity is most likely due to the intrinsic buffering capacity of cellular proteins as well as the activity of any proton pumps during ischemia.

There is considerable scatter in the relationship between the total lactate production and tissue pH at pH < 6.1. This is most likely due to the inherent uncertainty in pH measurements by ^{31}P-NMR spectroscopy in this pH range, because this is a portion of the pH titration curve that is significantly removed from the pK_a of P_i (6.8). Thus, a small difference (or error) in the chemical shift of P_i results in large changes in the measured pH.

It should be noted that although total cardiac lactate production was measured (i.e., lactate present in the intracellular and interstitial compartments, as well as in the perfusate surrounding the heart), we determined only the intracellular pH. This is appropriate when determining the cardiac buffering capacity, however. All protons produced as lactic acid by anaerobic glycolysis originate within the cytosol. Thus, total lactate production as measured in this study gives a record of the total number of protons that were added to the intracellular compartment during ischemia. Any protons produced during ischemia are then buffered by one of three mechanisms, (a) physico-chemical buffering, (b) reactions that consume or produce protons and (c) transfer of acid or alkali between the cytosol to organelles or the extracellular space [8]. Protons transported to the extracellular compartment represent cardiac buffering by this third mechanism, and contribute to intrinsic cardiac buffering as defined in this study.

Using the value for buffering capacity reported here, one can predict values of proton or lactate production during myocarial ischemia based on changes in pH, in this model of total global ischemia. This may be especially useful when used

in conjunction with ^{31}P-NMR spectroscopy, since one can monitor changes in intracellular pH by this technique. One must take into account the degree of ATP and PCr hydrolysis, but this can also be done by ^{31}P-NMR spectroscopy (see Appendix).

The fact that the intrinsic cardiac buffering capacity increases at lower pH may serve as a protective mechanism during myocardial ischemia. Jacobus and co-workers have demonstrated a depression of the left ventricular performance with decreasing intracellular pH during myocardial ischemia [26]. Furthermore, Neely and Grotyohann have shown that anaerobic glycolytic products, such as lactate and hydrogen ion, play a major role in recovery of the left ventricular function following ischemia and reperfusion [3]. Thus, a greater capacity for intracellular buffering at lower pH values may limit the degree of intracellular acidosis during ischemia, thereby limiting further functional deterioration and the extent of irreversible myocardial damage.

In summary, the relationship between total buffering capacity and pH varies considerably. At pH > 6.4, the total buffering capacity is 25 μmol H^+/g wet wt. per pH unit with the majority of cardiac buffering due to ATP/PCr hydrolysis and neutralization of bicarbonate and P_i. At pH < 6.4, the total buffering capacity increases progressively with an increasing proportion of cardiac buffering coming from intrinsic cardiac buffers, most likely from intracellular proteins. The increased intrinsic buffering capacity at lower pH values may limit the degree of intracellular acidosis and may protect the heart from irreversible damage during myocardial ischemia.

Appendix

Calculation of effective proton production from ATP and PCr hydrolysis and buffering by known cardiac buffers

The net hydrolysis of ATP either produces or consumes protons depending on the pH at which hydrolysis occurs [12]. When ATP hydrolysis is accompanied by a change in pH (e.g., during ischemia), the reaction can be represented by:

$$\begin{array}{lll} \text{pH 7.5} & \text{MgATP} + 3\text{H}_2\text{O} \rightarrow \text{AdoH} + 3\text{P}_\text{i} + \text{Mg}^{2+} & \\ & \searrow \downarrow & \\ \text{pH}_\text{f} & \text{AdoH} + 3\text{P}_\text{i} + \text{Mg}^{2+} & \text{(A-1)} \end{array}$$

where pH_f is the final pH of the ischemic tissue and AdoH is adenosine. Thus, to determine net proton production/consumption by ATP hydrolysis during ischemia, one can first calculate the proton production of ATP hydrolysis at pH 7.05 (ΔH^+_{ATP}) and then calculate the proton consumption ($\Delta H^+_{P_i}$) required to lower the pH from 7.05 to a new lower pH. Thus:

$$\Delta H^+/\text{mol ATP hydrolysis} = \Delta H^+_{ATP} - \Delta H^+_{P_i} \qquad \text{(A-2)}$$

Using the dissociation constants of Alberty [9], the hydrolysis of ATP to adenosine and inorganic phosphate at pH 7.05 can be expressed as:

$$\begin{bmatrix} 0.845\,\text{MgATP}^{2-} \\ + \\ 0.002\,\text{MgHATP}^{1-} \\ + \\ 0.085\,\text{ATP}^{4-} \\ + \\ 0.068\,\text{HATP}^{3-} \end{bmatrix} + 3\,\text{H}_2\text{O} \rightarrow \text{Ado}$$

$$+ \begin{bmatrix} 1.92\,\text{HPO}_4^{2-} \\ + \\ 1.08\,\text{H}_2\text{PO}_4^{1-} \end{bmatrix} + 0.847\,\text{Mg} + 0.99\,\text{H}^+ \qquad \text{(A-3)}$$

Thus, complete hydrolysis of ATP yields 0.99 equivalents of protons and 3 equivalents of P_i at pH 7.05.

The number of proton equivalents required to acidify the products of ATP hydrolysis to a lower pH is defined by the buffering of inorganic phosphate (adenosine has no significant changes in its ionization state from pH 5.5 to 7.5) and is expressed as:

$$\Delta H^+_{P_i} = P_T(f_f - f_i) \qquad \text{(A-4)}$$

where P_T is the total concentration of inorganic phosphate, f_f is the fraction of $H_2PO_4^{1-}$ (acid form of P_i) at the final lower pH and f_i is the fraction of $H_2PO_4^{1-}$ at the initial pH. Rearrangement of the Henderson-Hasselbalch equation and substitution into this expression yields:

$$\Delta H^+_{Pi} = -P_T\left(\frac{1}{10^{pH_f - pK}} - 0.36\right) \qquad \text{(A-5)}$$

where the pK of P_i is 6.8.

Thus, the net proton production/consumption from hydrolysis of ATP to adenosine during myocardial ischemia can be expressed as:

$$\Delta H^+/\text{mol ATP hydrolysis} = \Delta H^+_{ATP} - \Delta H^+_{P_i}$$

$$= 0.99 - 3\left(\frac{1}{1+10^{pH_f-6.8}} - 0.36\right) \quad \text{(A-6)}$$

where pH_f is the final pH of the ischemic tissue.

The balanced equation for ATP hydrolysis to AMP and 2 P_i at pH 7.05 may be written similarly to that for hydrolysis of ATP to 3 P_i.

$$\begin{bmatrix} 0.085\,ATP^{4-} \\ + \\ 0.845\,MgATP^{2-} \\ + \\ 0.068\,HATP^{3-} \\ + \\ 0.002\,MgHATP^{1-} \end{bmatrix} + 2H_2O$$

$$\rightarrow \begin{bmatrix} 0.8\,AMP^{2-} \\ + \\ 0.2\,HAMP^{1-} \end{bmatrix} + \begin{bmatrix} 1.28\,HPO_4^{2-} \\ + \\ 0.72\,H_2PO_4^{1-} \end{bmatrix} + 0.85\,Mg^{2+} + 1.15\,H^+ \quad \text{(A-7)}$$

Note that since AMP and P_i have a similar pK (6.5 vs. 6.8), the overall proton balance for this reaction is similar in comparison to the hydrolysis of ATP to adenosine and 3 P_i.

Further hydrolysis of adenosine to inosine or hypoxanthine will consume 1 mol of H^+ per mol of inosine or hypoxanthine formed and 2 mol of H^+ per mol of xanthine formed. For conversion of adenosine to inosine (or AMP to IMP) the net reaction may be represented by:

$$AdoH + H_2O + H^+ \rightarrow \text{inosine} + NH_4^+ \quad \text{(A-8)}$$

The net proton production/consumption expected from ATP hydrolysis will depend on the products formed upon hydrolysis. Eqn. A-9 describes the effects of the various products of ATP hydrolysis on the proton production.

mol H^+ produced/mol ATP hydrolyzed

$$= (f_{AdoH} + f_{ino} + f_{hypo} + f_{xan}) \times \left[0.99 - 3\left(\frac{1}{1+10^{pH-6.8}} - 0.36\right)\right]$$

$$+ (f_{AMP} + f_{IMP})\left[1.15 - 2\left(\frac{1}{1+10^{pH-6.8}} - 0.36\right) - \left(\frac{1}{1+10^{pH-6.5}} - 0.2\right)\right]$$

$$- (f_{ino} + f_{hypo} + f_{IMP}) - 2(f_{xan}) \quad \text{(A-9)}$$

where f_{AdoH}, f_{ino}, f_{hypo}, f_{AMP}, f_{IMP} and f_{xan} represent the mol of adenosine, inosine, hypoxanthine, AMP, IMP and xanthine formed per mol of ATP hydrolyzed. The first term on the right of this equation represents the proton production due to ATP hydrolysis to adenosine and three equivalents of inorganic phosphate (Eqn. A-6). The second term represents the formation of two inorganic phosphate equivalents and AMP and subsequent buffering by P_i and AMP. The final two terms represent proton consumption by ammonium ion formation due to the formation of deaminated purine bases. The consideration of alternate products of ATP hydrolysis effectively raises the pH at which no net protons are produced by ATP hydrolysis from 6.5 (Eqn. A-6) to approx. 6.7 (Eqn. A-9, Fig. 5A).

Net proton production from PCr hydrolysis

Phosphocreatine has a sufficiently low pK (4.8) for, at all pH values considered here, the molecule to be totally ionized. In addition, the stability constants for magnesium complex formation are sufficiently low that the formation of these complexes can be ignored. The balanced equation for PCr hydrolysis at pH 7.05 is given in Ref. 12

$$PCr^{2-} + H_2O \rightarrow Cr + \begin{bmatrix} 0.64\,HPO_4^{2-} \\ + \\ 0.36\,H_2PO_4^{1-} \end{bmatrix} - 0.36\,H^+ \quad \text{(A-10)}$$

where Cr is the concentration of creatine after PCr hydrolysis.

The negative stoichiometry coefficient for the proton denotes that at pH 7.05, the hydrolysis of PCr consumes 0.36 mol H^+/mol PCr hydrolyzed. Using the approach described for ATP hydrolysis at pH 7.05 followed by titration of the inorganic phosphate to a new lower pH, it can be shown that the number of protons produced by hydrolysis of PCr during an ischemic episode which re-

sults in a lower pH is given by

$$\text{mol } H^+ \text{ produced/mol PCr} = -\left(\frac{1}{1+10^{pH+6.8}}\right) \quad \text{(A-11)}$$

Note that the 0.36 equivalents of protons produced is mathematically cancelled by the inclusion of the phosphate buffering consideration of Eqn. 5.

Buffering by inorganic phosphate initially present in cardiac tissue

Cardiac tissue contains inorganic phosphate prior to ischemia which will also serve to provide some buffering capacity. The number of moles of protons consumed by this inorganic phosphate buffer on changing the pH from 7.04 to a new value is given by

$$\text{number of } H^+ \text{ produced/mol } P_i \text{ present} = -\left(\frac{1}{1+10^{pH+pK}} - 0.36\right) \quad \text{(A-12)}$$

The contribution to tissue buffering from this pool is simply the number of H^+ buffered/mol P_i present times the concentration of P_i initially present in the tissue.

Buffering by the bicarbonate buffer during ischemia

The globally ischemic myocardium may be considered a closed system in terms of the bicarbonate buffer, in which the total amount of dissolved CO_2 plus bicarbonate buffer are constant [8]. Using a wet wt./dry wt. ratio of 5.5, an extracellular volume of 0.46 ml/g wet wt., in intracellular volume of 0.36 ml/g wet wt. [27] $pCO_2 = 38$ mmHg intra- and extracellular, and pH in = 7.05, and pH out = 7.4, the total bicarbonate and dissolved CO_2 content of the pre-ischemic heart can be shown to be 15.8 μmol/g wet wt. of which 1.0 μmol/g wet wt. represents dissolved CO_2 and 14.8 μmol/g wet wt. represents bicarbonate. Using an effective pK_a of 6.1 for the bicarbonate buffer system, the amount of buffering available from the bicarbonate system as the pH changes from its pre-ischemic value to its post-ischemic value is given by

$$\mu\text{mol } H^+ \text{ produced/g wet wt.} = -15.8\left(\frac{1}{1+10^{pH-6.1}} - \frac{1}{1+10^{7.05-6.1}}\right) \quad \text{(A-13)}$$

where 15.8 represents the total bicarbonate and dissolved CO_2 of the tissue, 6.1 is the pK_a of the bicarbonate system and 7.05 is the initial pH of the intra- and extracellular compartment. This method in effect assumes that CO_2 does not leave the ischemic heart and that post ischemia, the bicarbonate also equilibrates between intra- and extracellular spaces. Although it is not known whether bicarbonate equilibrates across cellular membrane after ischemia or whether CO_2 can diffuse out of the ischemic myocardium, experimental observation of rapid pH compensation in cardiac tissue subjected to changes in extracellular pCO_2 and HCO_3^- [6] and increased buffering capacity as a consequence of inclusion of extracellular buffers in the perfusate [1] suggest that these assumptions are reasonable.

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