BUFFER POWER AND INTRACELLULAR pH OF FROG SARTORIUS MUSCLE

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ABSTRACT Intracellular pH (pH_i) and buffer power of frog muscle were measured using pH-sensitive microelectrodes under conditions used previously in energy balance experiments because pH strongly influences the molar enthalpy change for phosphocreatine splitting, the major net reaction during brief contractions. The extracellular pH (pH_e) of HEPES buffered Ringer's solution influenced pH_i, but change in pH_i developed slowly. Addition or removal of CO_2 or NH_3 from the extracellular solution caused a rapid change in pH_i. The mean buffer power measured with CO_2 was 38.4 $mmol.l^{-1}.pH\ unit^{-1}$ (±SEM 2.1, $n = 49$) and with NH_3 was 36.2 (±SEM 5.5, $n = 4$) at 20–22°C. At 5°C, in experiments with CO_2 the mean buffer power was 40.3 (±SEM 2.6, $n = 3$). For pH_i values above ~7.0, the observed buffer power was greater than that expected from the values in the literature for the histidine content of intracellular proteins, carnosine and inorganic phosphate in the sarcoplasm. The measured pH_i values were similar to those assumed in energy balance calculations, but the high measured buffer power suggests that other buffering reactions occur in addition to those included in energy balance calculations.

INTRODUCTION

Phosphocreatine splitting is the major net reaction during brief contractions of frog muscle. The amount of energy produced per mol of this reaction (ΔH, $kJ.mol^{-1}$) is ~40% greater at pH 7.5 than at pH 6.5 (25°C, pMg = 3, Woledge, 1973, Woledge and Reilly, manuscript in preparation). Since this reaction absorbs H^+ ions, buffer reactions occur and contribute to the overall ΔH. Thus intracellular pH (pH_i) and buffering are highly relevant to energy balance studies in which heat and work output is compared with the amount of energy explained by phosphocreatine splitting (explained energy = mols of reaction × ΔH). Values of pH_i ranging from 6.9 to 7.5 have been measured in amphibian skeletal muscle fibers under various conditions (reviewed by Roos and Boron, 1981). The experiments reported here were undertaken to measure the pH_i and buffer power under conditions like those used in energy balance studies.

Preliminary accounts of some of these results have been presented (Curtin, 1982, 1984).

METHODS

Experiments were done on isolated sartorius of frog, *R. temporaria*. For experiments at 20–22°C the Ringer's solution contained ($mmol.l^{-1}$) KCl 3.7, $CaCl_2$ 2.0, and either HEPES or $NaHCO_3$ 10, 23, or 50, plus NaCl to give a total NaCl plus buffer of 105. For the experiments at 5°C, it contained ($mmol.l^{-1}$) KCl 3.7, $CaCl_2$ 2.0, NaCl 93, and either HEPES or $NaHCO_3$ 20. The HEPES buffered solutions were equilibrated with 100% O_2 and the pH was adjusted with NaOH. In bicarbonate-containing solutions pH was determined by the percent CO_2 in the $CO_2 + O_2$ gas in equilibrium with it. In some experiments with HEPES buffer solutions, 10 $mmol.l^{-1}$ NaCl was replaced by NH_4Cl for measurement of buffer power. Muscles were kept for several hours in solution before the experiment began because this is the usual practice in energy balance experiments. During the experiment fresh solution flowed through the bath (vol. 1 or 5 ml) at a rate of 4 $ml.min^{-1}$ or more. Control experiments were done to check that CO_2 escape from bicarbonate + CO_2 buffered solutions did not alter the pH in the bath or its CO_2 content.

The pH-sensitive microelectrodes were either the recessed-tip design (Thomas, 1974), or the liquid-membrane type (Schulthess et al., 1981). They were calibrated with Ringer's solution buffered with HEPES or ADA at the appropriate temperature. The liquid membrane electrodes were checked to ensure that they were insensitive to CO_2. All experiments at 5°C were done with recessed-tip pH microelectrodes. Membrane potential was measured with a separate, conventional KCl-filled microelectrode inserted in the same cell. The values were less negative than −90 mV because in the Ringer's solution the K^+ concentration was higher and the osmolarity was lower than commonly used. To establish that both electrodes were in the same cell, a brief hyperpolarizing current (of 50 nA or less) was passed through the KCl-filled electrode and the output of the pH-sensitive electrode was checked. A change of more than 2 mV during the time the current was on was taken as showing that the electrodes were in the same cell. The pH-sensitive microelectrode was connected to a Varactor Bridge operational amplifier (311J; Analog Devices, Inc., Norwood, MA). After amplification, the outputs of the two microelectrodes were subtracted and sent to a chart recorder or digital oscilloscope (Explorer III, Nicolet Instruments Ltd., Madison, WI) to give a record of intracellular pH. The membrane potential and full output of the pH-sensitive microelectrode were recorded separately on other channels. A flowing-KCl electrode in the bath near the outlet was the reference. In some experiments a virtual earth circuit was used to prevent current passage through the reference.

Control experiments were done to assess the accuracy and precision of the measurements of pH_i. Repeat penetrations of the fiber were made at

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the same location with the same pH-microelectrode so that errors due to variation of the electrode sensitivity to pH or due to changes in pH_i or membrane potential caused by damage to the membrane during the previous penetration could be detected. For 14 fibers in two muscles the mean difference between the signals in the first and second penetration was −2.6 mV (±SEM 1.9), which is equivalent to an error of 0.04 pH units (±SEM 0.03). In other experiments the membrane potential was measured simultaneously at two sites on the same fiber with the microelectrodes separated by ~0.2 mm, the usual distance between pH- and membrane potential-measuring microelectrodes. Errors due to variation in membrane potential along the fiber could thus be assessed. For 15 fibers in two muscles, the mean of the unsigned differences was 2.7 mV (±SEM 0.5) which is equivalent to an error of 0.05 pH units (±SEM 0.01).

Buffer Power

Buffer power (β) is defined here as the following ratio: amount of H^+ added (or removed) per unit volume of cell water (units, $mmol.l^{-1}$) ÷ the change in pH. Buffer power was determined from the change in pH_i that occurred when the extracellular solution was changed from solution containing no permeant acid or base to one at the same pH containing either the permeant acid CO_2, or the permeant base NH_3. This method is described in detail elsewhere (Aickin and Thomas, 1975). For the reaction summarized $CO_2 \rightarrow H^+ + HCO_3^-$, the pK is 6.26 at 5°C, and 6.12 at 20°C. The solubility of CO_2 is 0.0665 $mol.l^{-1}.atm$ at 5°C and 0.0377 at 20°C (all values refer to solutions containing 0.15 $mol.l^{-1}$ NaCl, Edsall and Wyman, 1959). For the reaction, $NH_4^+ \rightarrow NH_3 + H^+$, the pK is 9.29 at 25°C, 0.15 $mol.l^{-1}$ NaCl (Christensen et al., 1976).

Calculating the Buffer Power of a Defined Mixture of Buffers

For a solution containing one buffer,

$$\beta = -2.3 \times \{[K_b \times C \times H/(K_b + H)^2] + H + (K_w/H)\}, \quad (1)$$

where β is buffer power, C is the total buffer concentration, H is H^+ ion concentration, K_b is the buffer dissociation constant, and K_w is the dissociation constant for H_2O (Perrin and Demsey, 1974). When pH is between 6 and 8 the last term is negligible. When the pH is equal to the buffer's pK, its buffer power is at its maximum. If C is much greater than the concentration H^+ and OH^-, this maximum β is -0.575 × C. The buffering power of a mixture is the sum of the individual values.

Eq. 1 was used to calculate β for a mixture of histidine, carnosine and phosphate, which are buffers known to exist in frog muscle. The following pK values were used (20°C): histidine, 6.15 (Christensen et al., 1976), carnosine, 6.78 (Deutsch and Eggleton, 1938), phosphate, 6.79 (Bates and Acree, 1945), and water, 14.167 (Kaye and Laby, 1973). The histidine concentration was calculated to be 36 $mmol.kg^{-1}$ wet weight of muscle assuming (*a*) that the 18% of the wet weight of muscle that is solids (Hill, 1965) consists of protein, and (*b*) that the histidine content of total protein is like that of myosin, tropomyosin and actin (Bendall, 1969). Eggleton and Eggleton's (1933) measurement of carnosine, 5.3 $mmol.kg^{-1}$ wet muscle (±SEM 0.4, n= 10) was used. The inorganic phosphate in resting frog sartorius muscle is 3.3 $mmol.kg^{-1}$ wet weight (±SEM 0.1, n = 74, Curtin and Woledge, 1979). These concentrations were converted for direct comparison with the microelectrode measurements, using the volume fraction 0.58 *g* intracellular water excluding sarcoplasmic reticulum and mitochondria per 1 *g* wet muscle (see Baylor et al., 1982), giving values of 62 histidine, 9.1 carnosine, and 5.7 phosphate ($mmol.kg^{-1}$ intracellular water excluding sarcoplasmic reticulum and mitochondria).

RESULTS

Buffer power (β) was evaluated from measurements of pH_e and ΔpH_i upon exposure to CO_2. For the example shown in Fig. 1 *A*, 0.831 $mmol.l^{-1}$ CO_2 produced a relatively rapid change, pH_i 6.83 to 6.72. The H^+ ion added to the cell amounted to 3.31 $mmol.l^{-1}$, so β is $3.31/0.11 = 30.1$ $mmol.l^{-1}.pH\ unit^{-1}$. After the initial rapid acidification, pH_i remained relatively constant. In some fibers there was a slow drift, but the direction was not consistent from fiber to fiber. The mean rate of this slow phase was not significantly different from zero (-0.03 pH $units.h^{-1}$, ±SD 0.31, n = 34) and did not correlate with either pH_i in HEPES buffer (r = 0.26) or with the pH_i reached at the end of the rapid acidification in CO_2-containing solution (r = 0.29). Extrapolation of the pH_i values back to the time of change to CO_2-containing solution to remove the effect of the slow drift did not

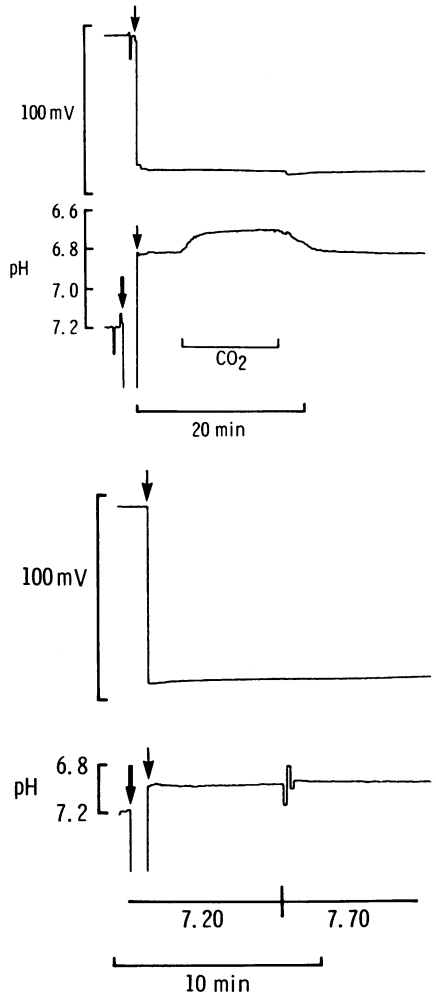

FIGURE 1 Digital oscilloscope records of membrane potential (*upper* trace) and pH (*lower* trace) from the same fiber in a frog sartorius muscle. Calibrations are shown at the left. The heavy arrows indicate when the pH-sensitive microelectrode was inserted and the light arrow insertion of the KCl-filled microelectrode. In *A* the pH of the extracellular solution was constant at pH 7.20. The solution was changed from one buffered with 10.0 $mmol.l^{-1}$ HEPES to one buffered with 10.0 $mmol.l^{-1}$ HCO_3^- and 0.831 $mmol.l^{-1}$ CO_2 (2.20% CO_2) as shown. In *B* the extracellular solution was buffered with 10.0 $mmol.l^{-1}$ HEPES (equilibrated with 100% O_2). The solution was changed from one at pH 7.20 to one at pH 7.70 as shown.

significantly alter the mean β (uncorrected 40.6 mmol.l^{-1}.pH unit^{-1}, ±SEM 2.7, and corrected 39.6 ±SEM 3.1 for the same group of 34 fibers). The values shown in Fig. 2 are based on the pH_i reached at the end of the rapid phase.

In these experiments a range of pH_e values was used to include that reported by Howell et al. (1970) for frog blood in vivo (pH 7.87 ±SEM 0.08 at 20°C), as well as the unphysiologically low pH_e values traditionally used with isolated muscle (as low as pH_e 7.0 at 5°C). As shown in Fig. 3, pH_i was correlated with the pH_e for fibers that had been in HEPES buffered solution at different pH values (20–22°C) for several hours. The regression line was calculated from the individual measurements on the 192 fibers from 39 muscles. The correlation between pH_e and pH_i is highly statistically significant (t = 10.5, P<0.01). This effect of pH_e on pH_i developed slowly. Fig. 1 *B* shows a typical record of pH_i during and after change of pH_e from 7.20 to 7.70 (HEPES buffered solution); there was little change in pH_i during the first few minutes in the new solution.

The buffer power values, determined from the acidification on exposure to CO_2, are summarized in Fig. 2; each point is a single measurements on a different cell. The mean for all the results is 38.4 mmol.l^{-1}.pH unit^{-1} (±SEM 2.1, n = 49, 20–22°C). The line in Fig. 2 shows the buffer power at 20°C, calculated as described in the Methods for the histidine, carnosine, and inorganic phosphate thought to exist in muscle. The experimental results do not indicate that buffer power was influenced by the buffer concentration or pH of the extracellular solution. In experiments on eight cells, CO_2 was repeatedly applied and removed while the microelectrodes remained in the cell. There was no consistent trend in successive measurements to suggest net movement of substances other than CO_2 across the cell membrane. In four cells β was measured by applying NH_4Cl in HEPES buffered solution (equilibrated with 100% O_2). This caused pH_i to move in the alkaline direction due to entry of NH_3 molecules into the cell which then combine with H^+. These values are shown Fig. 2 (mean 36.2 mmol.l^{-1}. pH unit^{-1}, ±SEM 5.5, n = 4) and are within the range of values measured with CO_2.

Measurements at 5°C on two muscles gave pH_i7.13 (±SEM 0.042, n = 13) in HEPES buffered solution and pH_i6.82 (±SEM 0.023, n = 20) in bicarbonate (20 mmol.l^{-1}) + CO_2 (2.3 mmol.l^{-1}) buffered solution at the same pH_e7.20. Experiments like that shown in Fig. 1 *A* were done on three cells in different muscles to determine β. The mean, 40.3 mmol.l^{-1}.pH unit^{-1} (±SEM 2.6, n = 3, range 35.1–43.0) was not different from that measured at room temperature. For 5°C the calculated β line in Fig. 2 would be shifted approximately 0.4 pH units in the alkaline direction because temperature affects the equilibrium constants of the buffer reactions.

DISCUSSION

In solution containing CO_2 at 20–22°C and at 5°C, pH_i was ~0.2 pH units more acid than it is in solution at the

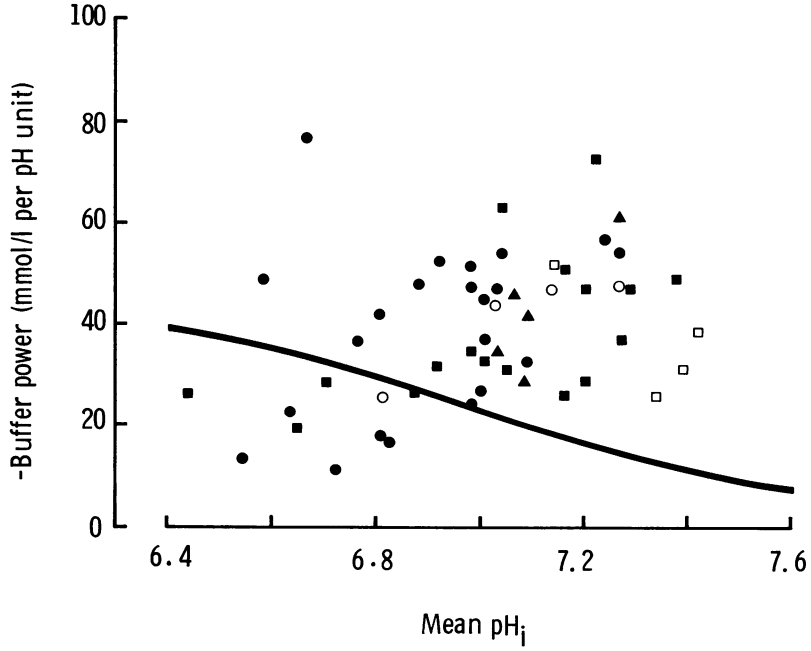

FIGURE 2 Values of buffer power plotted against the mean of the intracellular pH before and after it changed. Each point is for one fiber. The symbols indicate results of experiments in which the muscle was exposed to solution containing (mmol.l^{-1}) □, 10.0 NH_4Cl; ●, 10.0 HCO_3^- + 1.15 CO_2 (pH_e 7.06); ○, 10.0 HCO_3^- + 0.263 CO_2 (pH_e 7.70); ▲, 23.0 HCO_3^- + 1.29 CO_2 (pH_e 7.37); ■, 50.0 HCO_3^- + 1.15 CO_2 (pH_e 7.76). The line is the buffer power calculated for a mixture of histidine (pK6.15, 62 mmol.l^{-1}), carnosine (pK 6.78, 9.1 mmol.l^{-1}), and phosphate (pK 6.79, 5.2 mmol.l^{-1}, concentrations in intracellular water, excluding sarcoplasmic reticulum and mitochondria).

same pH, but containing HEPES buffer. Buffer power was determined from this effect of CO_2 on pH_i. Fig. 2 compares the observed values of buffer power with that expected for a mixture of the known intracellular buffers, having the *pK*'s and concentrations in the intracellular water, given in the legend. For more acid values of pH_i than about 7.0, the observations overlap with the calculated values, but at more alkaline pH values the observed buffering is more effective than this histidine, carnosine, phosphate mixture. It could be that the buffering by proteins is not equivalent to that of its constituent amino acids, as has been assumed here, because for example their *pK*'s may be altered by interactions within the protein. However the *pK*'s would have to increase by at least a whole unit to fit the observations. It is also possible that there is an additional intracellular buffer(s) with a *pK* greater than 7.0. Alternatively, there may be another process that removes H^+ from the sarcoplasm. The Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchange across the surface membrane described by Abercrombie et al. (1983) are too slow to have contributed significantly to the buffering observed here. Nevertheless, the existence of a process different than simple chemical buffering should be considered because the required concentration of extra chemical buffer is so high. The buffer power unaccounted for would require approximately 35 $mmol.l^{-1}$ of an unknown buffer with a *pK* of 7.2 to explain it. Intracellular organelles may contribute significantly to the buffering power.

Effects of Extracellular pH

The results in Fig. 3 show that pH_i is affected by the pH of the extracellular solution buffered with HEPES, but the change occurs slowly. The fact that pH_i does not change immediately (Fig. 1 *B*) agrees with results of earlier experiments in which only the immediate effects of pH_e on pH_i were investigated (Bolton and Vaughan-Jones, 1977; Abercrombie et al., 1983). The longer term effect is more relevant to studies of contraction in which a muscle or a fiber is equilibrated in vitro for hours before or during the experiment. The probability of the correlation between pH_i and pH_e in Fig. 3 being due to chance is extremely small ($P<0.01$). The low correlation coefficient ($r = 0.607$) shows that other factor(s), in addition to pH_e, contribute to determining pH_i in any individual fiber. The cause of the residual variation in pH_i (that not due to pH_e) is not known. Controls described in the Methods indicate that errors due to imprecision in measurements of pH_i and variation in membrane potential along the fiber are small compared to the observed systematic variation of pH_i with pH_e. The hypothesis that the variation depended on the membrane potential was tested, but there was no correlation ($r = 0.05$, $P = 0.5$, 183 degrees of freedom). The variation between individual fibers in a muscle could reflect differences among fibers types in amphibian muscle. The dependence of pH_i on pH_e may explain some of the variation between pH_i values previously reported for amphibian muscle (see Introduction).

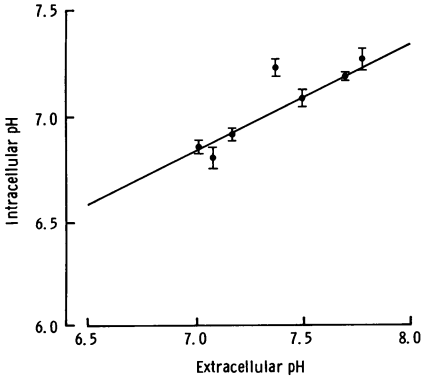

FIGURE 3 Measurements of pH_i of surface fibers of frog sartorius muscle (20–22°C) after several hours in HEPES buffered solution at various pH's. Symbols show the mean (±SEM). The line is a least squares fit to the individual measurements on 192 fibers in 39 muscles. $pH_i = 0.517\ pH_e + 3.22$ ($r = 0.607$, $t = 10.5$, $P<0.01$).

Implications for Energy Balance

The experimental conditions were chosen to duplicate those of previous energy balance studies (see Introduction). At 20°C with phosphate buffered Ringer's solution at pH_e 7.3 (Canfield and Marechal, 1973), pH_i was close to the value 7.0 assumed in energy balance calculations (Curtin and Woledge, 1978). A substantial error in the assumed pH_i (if the actual value is greater than 8.0) might have accounted for the apparent energy imbalance in these studies, since ΔH for phosphocreatine splitting is pH sensitive (Woledge, 1973; Reilly and Woledge, manuscript in preparation), but this appears to be ruled out.

In energy balance experiments at 0°C with bicarbonate (25 $mmol.l^{-1}$) at pH_e7.2, a value of pH_i7.0 was also assumed (Curtin and Woledge, 1979). In the present experiment, the lowest temperature achieved was 5°C, giving pH_i6.82 (±SEM 0.023, $n = 20$). This value would increase the calculated energy gap by ~10%, using a ΔH for phosophocreatine splitting of -30 $kJ.mol^{-1}$ at pH 7.00 and -28 $kJ.mol^{-1}$ at pH 6.82 (Fig. 8 of Curtin and Woledge, 1978). Thus the observations of pH_i indicate that some other explanation must be sought to account for these energy gaps.

The unexpectedly large buffer power observed in this study suggests that unidentified H^+ ion reactions, or uptake and release from intracellular organelles, are taking place. Since these processes may occur during contraction and would involve energy changes, they should themselves be considered as possible candidates to account for the unexplained energy production during contraction.

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