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THE INFLUENCE OF MUSCLE RESPIRATION AND GLYCOLYSIS ON SURFACE AND INTRACELLULAR pH IN FIBRES OF THE RAT SOLEUS

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SUMMARY

1. Extracellular pH (pH_o) and intracellular pH (pH_i) of superficial fibres of the rat soleus muscle were measured *in vitro* using pH-sensitive glass micro-electrodes. The origin of the pH gradient existing between the bulk phase of extracellular solution and the surface of muscle fibres was investigated.

2. The pH_o decreased almost linearly over a distance of 285 μm from bulk solution to fibre surface. The magnitude of the bulk–surface pH gradient is greater in the mid region of the muscle than close to the tendon.

3. Decreasing the superfusate velocity increased the magnitude of the pH gradient. Reducing the buffer capacity of the superfusing solution had the same effect.

4. Inhibiting the aerobic metabolism or stimulating it acidified the fibre surface. Inhibiting glycolysis alone, or both aerobic metabolism and glycolysis, alkalinized the fibre surface.

5. Inhibiting the membrane ionic exchange process involved in pH_i regulation had no effect on surface pH.

6. Changing the rate of aerobic or anaerobic metabolism quickly modified pH_i in most cases.

7. In conclusion the bulk–surface pH gradient seems to result mainly from diffusion of CO_2 and lactic acid across an unstirred layer of fluid covering the surface of muscle fibres.

INTRODUCTION

Micro-electrodes were first used in 1958 by Caldwell to measure pH in superficial fibres of isolated crab muscle *in vitro*. With sharper micro-electrodes it has recently been shown that surface pH (pH_s) is 0·1–0·2 unit lower than the pH of the bulk solution (pH_b) in the rat soleus under resting conditions (de Hemptinne, 1980). In the present work, the factors contributing to the development of a pH gradient between the bulk phase and the surface of the muscle cells were analysed in greater detail. In order to investigate the role of an unstirred layer effect, we measured the build up of the pH gradient as a function of the superfusion velocity, the buffering capacity of the superfusion solution and the location of the micro-electrode on the

muscle. To examine the role of the cellular production of diffusible acids, we investigated the influence of a variety of metabolic conditions on both pH_s and intracellular pH (pH_i). Finally, we inhibited the membrane ionic exchange process involved in pH_i regulation in order to evaluate its own contribution. Some of these results have been reported in preliminary form (de Hemptinne & Huguenin, 1982).

METHODS

The methodology was the same as described previously (de Hemptinne, 1980). Briefly, measurements were performed on isolated rat soleus muscle. The preparation was carefully cleaned of its connective and adipose tissue, pinned in a chamber and superfused at 2·4 bath volumes per minute. The Ringer solution was gassed with nominally 5% CO_2/95% O_2. The composition of the control solution (mM) was: NaCl, 121; KCl, 4; $CaCl_2$, 10; $MgCl_2$, 1; $NaHCO_3$, 21; Na_2HPO_4, 0·1; glucose, 10; its pH was 7·3–7·4 at 37 °C. HEPES-buffered solutions were made by replacing $NaHCO_3$ in the Ringer solution with NaCl and titrating the solution which contained either 2, 5, 10 or 20 mM-HEPES with concentrated NaOH to the desired pH at 37 °C. These solutions were gassed with 100% O_2. Single-barrelled glass micro-electrodes with a round tip (diameter 30–50 μm) were used to measure extracellular pH. The pH_i was determined using double-barrelled recessed-tip micro-electrodes (tip diameter $\simeq$ 1 μm). The electrical arrangements have been described previously. When necessary, the chamber temperature could be reduced from 37 °C to 8 °C; 90% of the temperature jump took place within 5 min. Results were expressed as $\bar{x} \pm$ S.E. of mean. Differences were considered statistically significant for $P < 0{\cdot}05$ (Student's t test). Each experiment was performed on at least four different preparations; n indicates the number of measurements.

RESULTS

Surface pH: dependence on location

The pH recorded at the surface of superficial muscle fibres at the level of the muscle belly was lower than in the bulk phase of the superfusate (Fig. 1). The mean magnitude of the bulk–surface pH gradient as measured in sixteen muscles was $0{\cdot}161 \pm 0{\cdot}012$ pH unit. The relation between the radial distance from the surface of the muscle cells and pH was practically linear and extended over a mean distance of 285 ± 26 μm ($n = 16$). This observation was not a mechanical artifact and did not depend on diameter or geometry of the micro-electrodes. The isolated soleus superfused longitudinally *in vitro* is therefore covered by an acid layer within which no voltage gradient could be seen.

In some experiments the pH electrode was also placed at the very end of the muscle close to the tendon where, as a result of tapering of the muscle fibres, muscle thickness is reduced to a single layer of cells. At this location, surface acidity is also present, as shown in Fig. 2*A*, but significantly less than on the muscle belly. On the tendon variable results were obtained. In some cases a slight surface acidity could be recorded; in other cases pH_s was equal to pH_b.

Surface pH: dependence on the buffering capacity of the medium and superfusion velocity

Fig. 2*B* shows that surface acidity increases significantly and reversibly when the buffering power of the superfusion solution is decreased by reducing the concentration of HEPES from 20 to 2 mM at constant pH. With intracellular pH micro-electrodes we also measured a significant intracellular acid change ($0{\cdot}120 \pm 0{\cdot}023$ ΔpH; ($n = 5$)

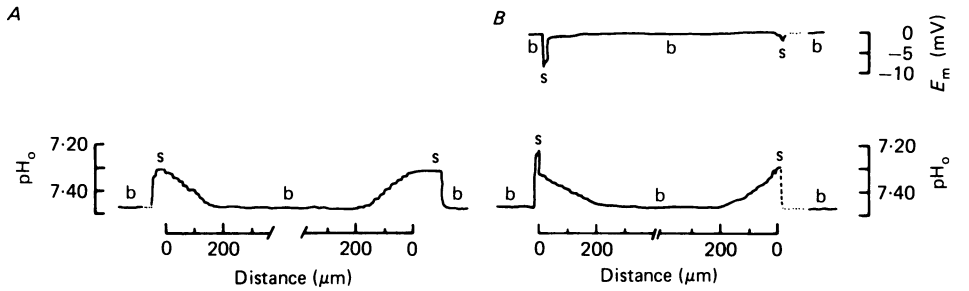

Fig. 1. Relation between extracellular pH (pH_o) and distance in the vicinity of the fibre surface. The distance indicates how far the micro-electrode tip lay away from the fibre surface. The labels 'b' and 's' represent bulk solution and fibre surface respectively. The pH was successively measured with a single-barrelled (*A*) and a double-barrelled (*B*) micro-electrode. The upper trace in *B* gives the voltage recorded by the reference barrel of the micro-electrode (E_m).

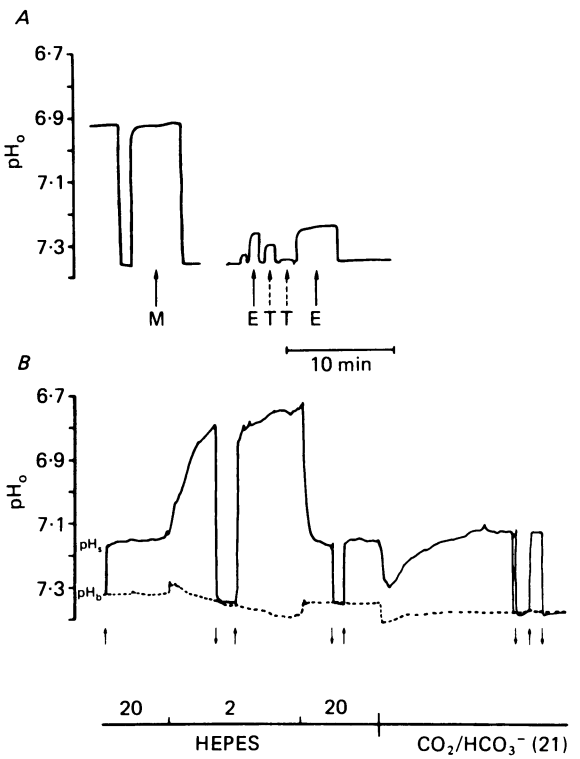

Fig. 2. Surface pH (pH_s) and bulk pH (pH_b) as measured in the presence of an isolated and superfused rat soleus muscle. *A*: at M the micro-electrode measures pH_s at the mid part of the muscle (on the muscle belly); at E the micro-electrode is localized at the end part of the muscle (close to the tendon); at T the micro-electrode is localized at two different places on the tendon. *B*: the superfusion solution is buffered with either 20 or 2 mM-HEPES or as indicated with 21 mM-HCO_3^-/5 % CO_2. The continuous line gives pH_s when the micro-electrode is pressed against the surface of the muscle (at arrows pointing upwards). At arrows pointing downwards, the micro-electrode is withdrawn from surface to bulk. The interrupted line gives pH_b as measured with a second pH-sensitive micro-electrode.

on switching from 20 to 2 mM-HEPES; this followed the extracellular pH (pH_o) change.

The pH_s was slightly lower in 21 mM-HCO_3^--buffer than in 20 mM-HEPES. The transient increase in pH_s seen on switching from HEPES to CO_2/HCO_3^- can be attributed to CO_2 penetrating the cells faster than HCO_3^-, which remain in relative

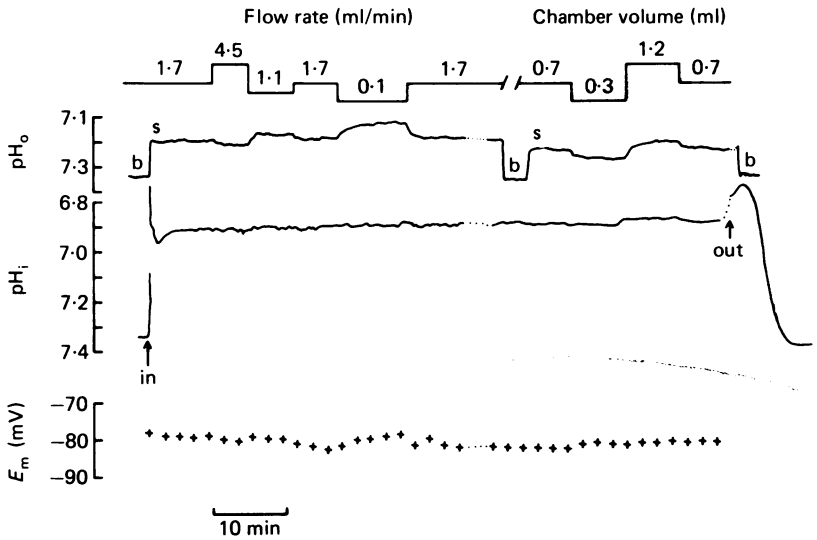

Fig. 3. Effect of flow rate and chamber volume on pH_s, pH_i and resting potential (E_m). The chamber volume was varied by moving upwards and downwards the paraffin layer covering the superfusate. At 'b' (upper trace) the tip of the extracellular micro-electrode was lying in the bulk solution; at 's' it was positioned on the fibre surface. At 'in' (middle trace) one fibre was impaled; at 'out' it was disimpaled. The dotted lines indicate a break of several minutes in the record. In this and subsequent Figures the pH_o and pH_i traces are slightly out of phase. This was to allow the pens on the recorder to move freely past each other.

excess with respect to the concentration of the local dissolved CO_2 in the unstirred surface layer of fluid surrounding the muscle. Similar pH_s transients were observed during transmembrane influx of weak organic acids (Marrannes, de Hemptinne & Leusen, 1981).

Fig. 3 is an original recording which illustrates the effect of varying the superfusion velocity. Two procedures were used: first, the flow rate through the chamber was altered but the chamber volume kept constant; secondly, the chamber volume was altered at constant flow rate. Reducing the superfusion velocity lowered pH_s while increasing it had a slight opposite effect. Thus, increasing the flow rate from 2·4 to 6·4 bath volumes per minute raised pH_s by $0{\cdot}031 \pm 0{\cdot}004$ pH unit ($n = 9$); decreasing it from 2·4 to 0·1 bath volumes per minute lowered pH_s by $0{\cdot}060 \pm 0{\cdot}003$ pH unit ($n = 4$). The pH_i and resting potential showed little or no change.

Fig. 4*A* displays the effect of simulating ischaemia by arresting the superfusion and having the muscle immersed in paraffin oil. This condition is of potential interest since it more or less reproduces an important physiopathological situation. Within

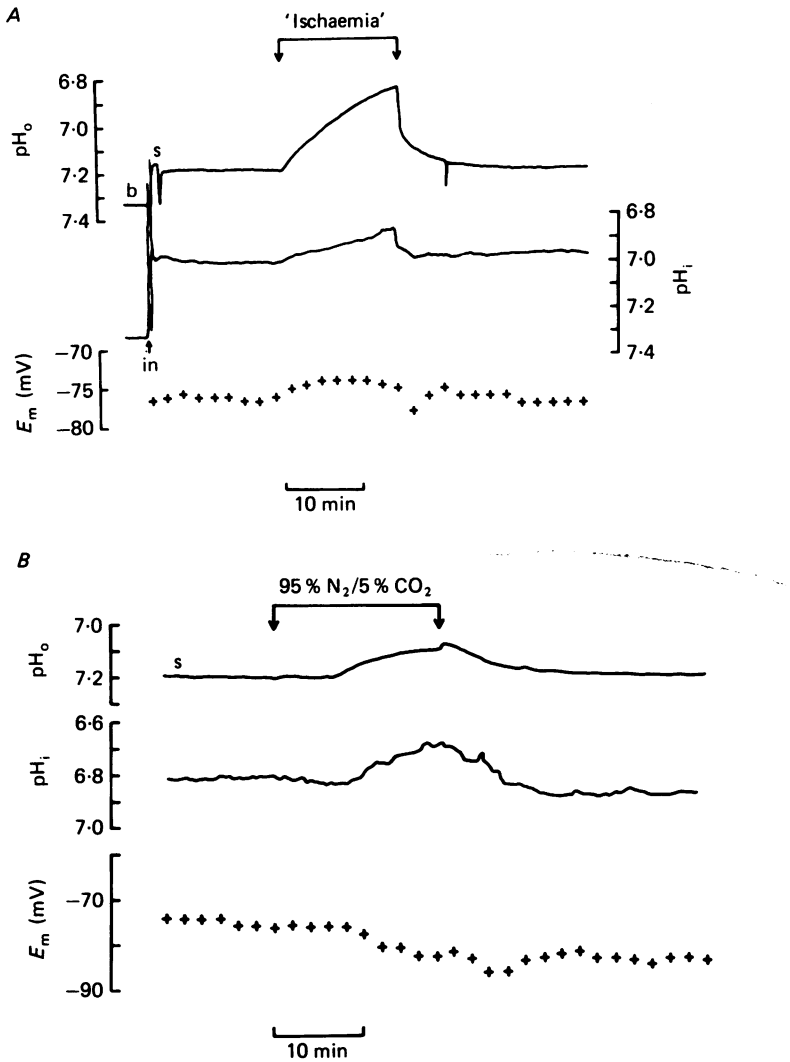

Fig. 4. *A*: ischaemia was simulated *in vitro* by stopping superfusion and moving downwards the paraffin layer covering the superfusate so as to give only a 100–200 μm thick layer of Ringer at the muscle surface. Labels 'b' and 's' indicate respectively bulk solution and fibre surface. At 'in' a fibre was impaled. *B*: the effect of equilibrating Ringer with 95% N_2/5% CO_2. Due to leakage of O_2 from ambient air to saline, the P_{O_2} of the solution inside the experimental chamber was 40 mmHg.

10–20 min, pH_s and pH_i both decreased to values ranging from 6·8 to 7·0, while the resting potential (E_m) dropped to between −56 and −87 mV during the 'ischaemic period'. The rate of decrease of pH_s was $2{\cdot}36 \pm 0{\cdot}55 \times 10^{-2}$ unit/min, of pH_i $0{\cdot}77 \pm 0{\cdot}20 \times 10^{-2}$ unit/min and of the resting potential $0{\cdot}73 \pm 0{\cdot}17$ mV/min ($n = 6$). The pH_s, pH_i and resting potential quickly returned to their starting level on re-perfusing the muscle.

Effect of inhibiting or stimulating muscle respiration

Cyanide. This drug inhibits aerobic metabolism and stimulates anaerobic reactions. After about 10 min of application it reversibly and markedly lowered pH_s and slightly lowered pH_i (Fig. 5*A*). Initially, however, we observed a transient alkalinization of both pH_s and pH_i on application of NaCN, and a transient acidification on its removal. The resting potential sometimes decreased (Fig. 5*A*) but more often increased as a result of application of NaCN.

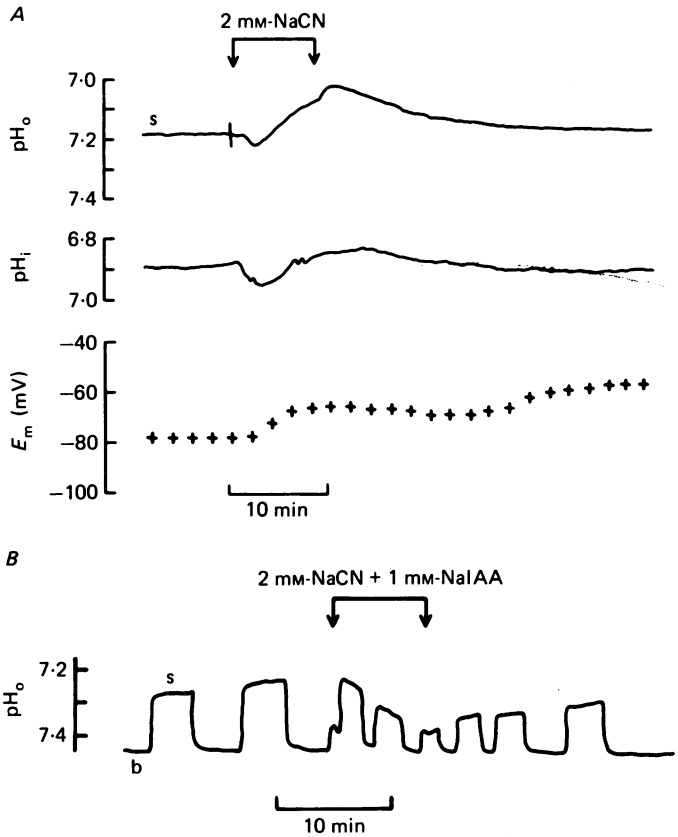

Fig. 5. Effect of NaCN alone (*A*) and of NaCN and Na iodoacetate (NaIAA: *B*). In *B*, the extracellular micro-electrode was repetitively switched from bulk solution (b) to fibre surface (s). This allowed supression of the movement artifacts which were always present during continuous recording of surface pH under these conditions.

Hypoxia. This condition interferes with normal cellular respiration; it is also of physiopathological interest. Fig. 4*B* illustrates its effect. After an initial delay, a progressive acidification appeared at the fibre surface and intracellularly, as well as a hyperpolarization. These effects were usually reversible within 10 min after the end of hypoxia. In some cases a transient acidification appeared at the fibre surface and intracellularly immediately after termination of hypoxia.

Dinitrophenol. This substance uncouples oxidative phosphorylation. At the concen-

tration of 0·02 mM as used here, it produces a 2-fold stimulation of state-respiration in rat liver mitochondria (Hemker, 1964). It also acts as a proton carrier across biological membranes. Fig. 6*A* illustrates its effect: surface acidification associated with decrease of pH_i and hyperpolarization. Higher concentrations of dinitrophenol (0·1 and 1·0 mM) caused an extremely fast acidification of fibre surface and intracellular fluid, and hyperpolarization.

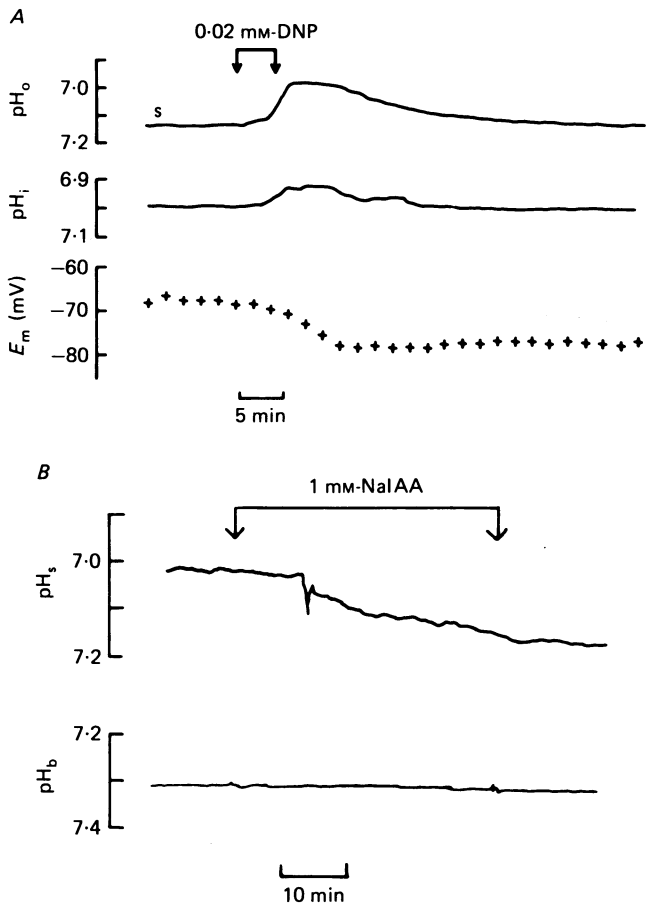

Fig. 6. Effect of dinitrophenol (DNP: *A*) and of Na iodoacetate (NaIAA: *B*). In *B*, the short-lasting deflexion of pH_s in presence of NaIAA is presumably due to a movement of the preparation.

Effect of inhibiting muscle glycolysis

In presence of Na iodoacetate, an inhibitor of glycolysis, pH_s became more alkaline by $0·10 \pm 0·01$ unit ($n = 4$) within 40 min (Fig. 6*B*). This effect was irreversible. No reliable recording of pH_i and resting potential could be obtained, presumably because of movement of the muscle which goes into rigor.

Effect of inhibiting muscle respiration and glycolysis

Cyanide and iodoacetate. Muscle respiration and glycolysis were simultaneously inhibited by administration of these two substances (Fig. 5*B*). The fibre surface was alkalinized by 0·08±0·01 pH unit ($n = 6$) within 10 min. On removal of the inhibitors, the effect increased further in four experiments and was partially reversed in two other experiments. No statistically significant pH_s change was present at the twentieth minute following inhibitor removal. No satisfactory recording of pH_i and resting potential could be made because of rigor.

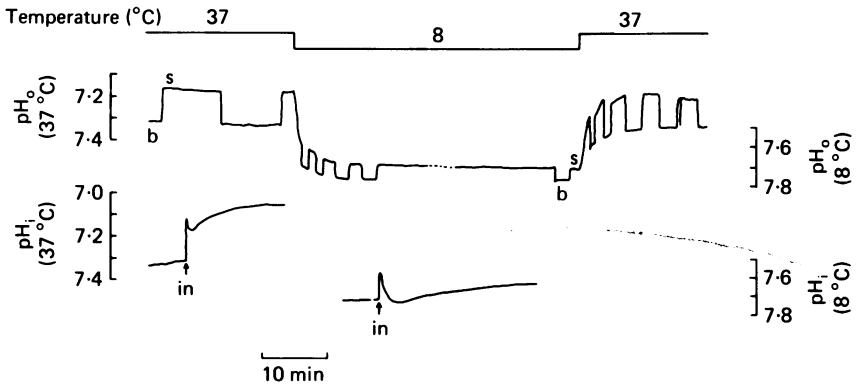

Fig. 7. The effect of temperature on pH_o and pH_i. This experiment was performed in CO_2/HCO_3^--free, HEPES-buffered Ringer. Note the different pH scales at 37 °C (left) and 8 °C (right). Middle trace: the extracellular micro-electrode was often switched from bulk solution (b) to fibre surface (s). The dotted line indicates a break of several minutes in the record. Bottom trace: the micro-electrode tip was initially lying in the bulk solution. At 'in' the muscle fibre was impaled.

Hypothermia. Lowering the temperature slows all metabolic processes, in particular muscle respiration and glycolysis. In order to simplify the preparation of solutions, an iso-osmotic CO_2/HCO_3^--free Ringer was used. It was buffered to pH 7·3–7·4 at 37 °C with 10 mM-HEPES and its osmolarity was adjusted by addition of NaCl and sucrose. Since both the pH of solutions and the pH-sensitivity of micro-electrodes are a function of temperature, it was necessary to calibrate micro-electrodes at 37 °C and at 8 °C, and to use a different pH scale for each temperature. The HEPES-buffered Ringer had a pH of 7·7–7·8 at 8 °C. Lowering the temperature from 37 to 8 °C markedly increased pH_b and pH_s (Fig. 7). However, pH_s increased more than pH_b and therefore the bulk–surface pH gradient decreased from 0·22±0·02 unit ($n = 8$) at 37 °C to 0·08±0·02 unit at 8 °C. This effect was quickly and fully reversible when temperature was increased from 8 to 37 °C.

Fig. 7 also illustrates the effect of temperature on pH_i. One fibre was impaled at 37 °C and another at 8 °C. Lowering temperature raised pH_i by some 0·6 pH unit. The average pH_i values were 7·12±0·04 ($n = 23$) at 37 °C and 7·65±0·04 ($n = 15$) at 8 °C. Another effect of lowering temperature was to reduce the bulk–intracellular pH gradient. This gradient was 0·23±0·03 unit ($n = 23$) at 37 °C and only 0·09±0·04 unit ($n = 15$) at 8 °C. Cooling the muscle fibres had no significant effect on resting

potential, which was $-63{\cdot}7 \pm 2{\cdot}3$ mV ($n = 23$) at 37 °C and $-60{\cdot}7 \pm 2{\cdot}9$ mV ($n = 15$) at 8 °C. However, impaling fibres at low temperature caused a slow progressive depolarization which was reversed at 37 °C.

Effect of inhibiting the ionic regulation of pH_i

The simultaneous application of 0·1 mM-amiloride and 0·1 mM-4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) is known to block the membrane ionic exchange process which underlies recovery of pH_i seen after acid loading the cells (Aickin & Thomas, 1977*b*). Under steady-state conditions these substances had no noticeable effect on pH_s.

DISCUSSION

The observations described in this paper show that muscle metabolism influences pH_s, pH_i and resting potential.

Influence of muscle metabolism on pH_s

The observations made under our experimental conditions can be explained by the existence of an unstirred layer of fluid surrounding the preparation. CO_2 produced by oxidative metabolism of the cells steadily diffuses from the intracellular to the extracellular compartment, entering an unstirred layer covering the fibre surface. Some CO_2 molecules react with water and dissociate to HCO_3^- and H^+ ions that diffuse along their concentration gradient. Other weak acids, e.g. lactic acid, produced by glycolysis and released into the extracellular compartment, cross the unstirred layer by essentially the same mechanism. This concept is consistent with most of our observations: (a) pH_s is lower than pH_b (Fig. 1), the difference being larger at the level of the muscle belly than close to the tendon and influenced by the buffering capacity of the superfusion fluid; (b) a pH gradient is present over an average distance of 285 μm; (c) increasing the superfusion velocity and therefore the stirring of bulk solution raises pH_s slightly; (d) mimicking ischaemia lowers pH_s, presumably by promoting accumulation of CO_2 and lactic acid at the fibre surface; (e) stimulating oxidative metabolism and therefore increasing CO_2 efflux lowers pH_s; (f) inhibiting glycolysis and thus reducing lactic acid efflux raises pH_s; (g) inhibiting oxidative metabolism and glycolysis decreases the bulk–surface pH gradient. On inhibiting oxidative metabolism, one could expect pH_s to rise as a result of reduced CO_2 efflux. In fact after the initial transient change, pH_s is lowered (Figs. 4*B* and 5*A*). This is not inconsistent with the model if one considers that under these circumstances anaerobic glycolysis is stimulated; the increased efflux of lactic acid would then exceed the effect of a reduced CO_2 efflux.

The present explanation agrees with recent studies on the diffusion of CO_2 across thin solution layers (Gros, Moll, Hoppe & Gros, 1976; Meldon, 1980) and on the diffusion of weak acids across lipid bilayer membranes (Gutknecht & Tosteson, 1973; Roos, 1975; Gutknecht, Bisson & Tosteson, 1977). The presence of a pH gradient without any associated voltage gradient implies a proton flux from fibre surface to bulk solution. Such a flux has actually been measured in isolated rat diaphragm by Cechetto & Mainwood (1978). This scheme does not consider the possible contribution

to the low pH_s of fixed negative charges on the outer surface of the cell membrane and meshwork of connective tissue covering the cells (de Hemptinne, 1980). It is, however, doubtful whether fixed negative charges can contribute to a pH gradient which would be measurable with our micro-electrodes. The length over which fixed surface charges can influence the activity of ions in the vicinity of the charge (Debye length) is of the order of 1 nm at the ionic strength of the physiological solutions. A similar gradient should also exist for other positively charged ions such as Na^+. Using Na^+-sensitive micro-electrodes we have never measured a gradient of Na^+ at the surface of the muscle.

We therefore conclude that the influence of muscle respiration and glycolysis on pH_s results to a large extent from chemical reactions associated with diffusion of CO_2 and metabolic (lactic) acids across an unstirred layer. This is consistent with observations by Dubuisson (1937) and Distèche (1960). A low pH_s is not restricted to rat soleus but has been reported in other preparations (Caldwell, 1958; Stegeman, 1964; Steinhagen, Hirche, Nestle, Bovenkamp & Hasselmann, 1976; Marrannes *et al.* 1981; Kost, 1982). The postulated unstirred layer is about ten times thicker than single muscle fibres. It is questionable whether in living muscle perfused via capillaries, a similar pH gradient exists between cell surface and blood. This seems to be the case, because measurements on perfused muscle with larger electrodes (tip diameter 50–400 μm) clearly show such a gradient (Steinhagen *et al.* 1976; Kost, 1982).

It should also be pointed out that the degree of surface acidity varies as a function of the buffering capacity of the buffer present in the superfusion solution. The depressive effect of HEPES (5 mM), as compared with CO_2/HCO_3^- (5%/12 mM), on the contractile performance of cardiac papillary muscles reported recently by Bay, Pelzer & Trautwein (1982) may perhaps to some extent depend on differences in pH_s.

Influence of muscle metabolism on pH_i

Aerobic metabolism and glycolysis influence pH_i in a complex way. Several mechanisms are involved, such as the production of CO_2 and organic (lactic) acids. The hydrolysis of ATP and creatine phosphate also play a role (Gadian, Radda, Dawson & Wilkie, 1982). A detailed discussion of these mechanisms is beyond the scope of the present paper.

Our observations show that inhibition of respiration (Figs. 4*A*, *B* and 5*A*) quickly lowers pH_i. Dinitrophenol has a similar effect (Fig. 6*A*). It might act either by stimulating respiration or by increasing passive influx of H^+ (Thomas, 1974, 1978). It is unclear to what extent each mechanism contributes to the present finding. In striking contrast with rat soleus, frog muscle shows, at a pH_o of 7·4, practically no pH_i decrease with 0·1–0·2 mM-dinitrophenol (Kostyuk & Sorokina, 1961). This difference is possibly due to the higher metabolic activity of rat soleus.

The effect of hypothermia on pH_i and bulk–intracellular pH gradient presumably results from several mechanisms: a slowing down in production of CO_2 and lactic acid; a shift in pK'_a of intracellular and extracellular buffers (Reeves & Malan, 1976); a possible proton redistribution between cytosol and intracellular organelles (Adler, 1972); and a decrease of active H^+ extrusion across sarcolemma (Aickin & Thomas, 1977*b*). It is noteworthy that the CO_2/HCO_3^- buffer is not involved in our

observations which were made in HEPES buffer. In the presence of CO_2/HCO_3^-, pH_i of mouse soleus muscle also increases in hypothermia (Aickin & Thomas, 1977*a*).

Typical changes of membrane potential appeared in our experiments. The hyperpolarization observed with hypoxia and dinitrophenol might result from an increase in K^+ conductance associated with raised intracellular concentration of Ca^{2+} (Wettwer, Hase & Lüttgau, 1980). The depolarization in simulated ischaemia could be due to accumulation of K^+ in the extracellular medium (Jennische, Hagberg & Haljamae, 1982).

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