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CHANGES IN MYOPLASMIC pH AND CALCIUM CONCENTRATION DURING EXPOSURE TO LACTATE IN ISOLATED RAT VENTRICULAR MYOCYTES

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SUMMARY

1. We investigated the mechanisms involved in the rise of myoplasmic calcium concentration ($[Ca^{2+}]_i$) when isolated rat ventricular myocytes were exposed to lactate. The intracellular pH (pH_i) and $[Ca^{2+}]_i$ were measured using the fluorescent indicators 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and fura-2, respectively. Cell shortening was used as a measure of contractile performance.

2. Exposure to 20 mM lactate at the normal extracellular pH (pH_o 7·4) for 10 min caused the pH_i to fall rapidly by 0·24 pH units and cell shortening was reduced. Thereafter, pH_i partially recovered by 0·16 pH units, which was paralleled by a recovery of shortening.

3. Exposure to lactate at a reduced extracellular pH (pH_o 6·4) induced a very large acidosis of 0·70 pH units and cell shortening was abolished. During maintained exposure to lactate the pH_i remained constant and cell shortening did not recover.

4. Application of Na^+–H^+ exchanger inhibitors, amiloride or ethylisopropyl-amiloride (EIPA), abolished the recovery of pH_i and shortening during maintained exposure to lactate at pH_o 7·4 and caused an additional acidosis during maintained application of lactate at pH_o 6·4.

5. Application of lactate at both the normal and reduced pH_o resulted in a rapid, followed by a slower, rise in $[Ca^{2+}]_i$. The diastolic and systolic $[Ca^{2+}]_i$ and the amplitude of the systolic rise in the $[Ca^{2+}]_i$ (the Ca^{2+} transient) all increased in both the rapid and the slow phase.

6. When lactate was applied at pH_o 7·4, in the presence of EIPA, the initial rise of $[Ca^{2+}]_i$ still occurred but the slower increase was abolished. This suggests an involvement of the Na^+–H^+ exchanger in the slower rise of $[Ca^{2+}]_i$.

7. In conclusion, the Na^+–H^+ exchanger is an important regulator of pH_i during a lactate-induced intracellular acidosis. The rise of $[Ca^{2+}]_i$ involves at least two mechanisms: (i) a rapid component which may represent reduced myoplasmic Ca^{2+} buffering, impaired Ca^{2+} removal by the sarcoplasmic reticulum or a direct inhibitory effect of protons on the Na^+–Ca^{2+} exchanger; (ii) a slower component linked to stimulation of Na^+–H^+ exchange which causes an increased $[Na^+]_i$ and stimulates the Na^+–Ca^{2+} exchanger, resulting in an enhanced Ca^{2+} influx.

INTRODUCTION

During early myocardial ischaemia there is an increase in the myoplasmic free calcium concentration ($[Ca^{2+}]_i$) (for review see Lee & Allen, 1991). This rise in $[Ca^{2+}]_i$ may contribute to the changes in action potential configuration and arrhythmias which occur during early ischaemia (Clusin, Buchbinder & Harrison, 1983). The aim of the present study was to investigate one of the cellular mechanisms responsible for this rise in $[Ca^{2+}]_i$.

A number of lines of evidence suggest that the elevated $[Ca^{2+}]_i$ during ischaemia may be caused by the acidosis which is known to accompany ischaemia. Firstly, an intracellular acidosis produced either by CO_2 (Allen & Orchard, 1983; Orchard, 1987; Harrison, Frampton, McCall, Boyett & Orchard, 1992), by lactate (Allen, Lee & Smith, 1989), or by NH_4Cl withdrawal (Kim & Smith, 1988; Kohmoto, Spitzer, Movsesian & Barry, 1990) causes an increase in the magnitude and duration of the Ca^{2+} transient which is comparable to that observed in ischaemia. Secondly, glycogen depletion in cardiac muscle, which reduces lactate production and the acidosis in ischaemia, also reduces the increase in $[Ca^{2+}]_i$ during ischaemia (Allen *et al.* 1989).

Several mechanisms have been proposed whereby protons could lead to an elevation of the $[Ca^{2+}]_i$ (for review see Orchard & Kentish, 1987). These include: (i) stimulation of the Na^+–H^+ exchanger resulting in a Na^+ influx (Deitmer & Ellis, 1980; Piwnica-Worms, Jacobs, Horres & Lieberman, 1985; Kaila & Vaughan-Jones, 1987; Harrison *et al.* 1992) which subsequently elevates $[Ca^{2+}]_i$ due to net Ca^{2+} influx on the Na^+–Ca^{2+} exchanger (Bers & Ellis, 1982; Kim & Smith, 1988; Bountra & Vaughan-Jones, 1989; Harrison *et al.* 1992); (ii) competition with $[Ca^{2+}]$ binding to myoplasmic Ca^{2+} buffers so that Ca^{2+} is displaced into the myoplasm (Bers & Ellis, 1982; Vaughan-Jones, Lederer & Eisner, 1983); (iii) impairment of Ca^{2+} uptake into the sarcoplasmic reticulum (SR) due to inhibition of the SR Ca^{2+} pump (Mandel, Kranias, De Gende, Sumida & Schwartz, 1982); (iv) inhibition of the trans-sarcolemmal Ca^{2+} efflux by direct inhibition of the Na^+–Ca^{2+} exchanger (Earm & Irisawa, 1986).

In the present study we examined the mechanism(s) responsible for changes in $[Ca^{2+}]_i$ during a lactate-induced intracellular acidosis in single ventricular myocytes. This procedure may more closely resemble the ischaemia-induced acidosis, caused by endogenous lactic acid production, than the acidosis produced with CO_2 or by NH_4Cl withdrawal. Our findings suggest that the elevation of $[Ca^{2+}]_i$ caused by an intracellular acidosis involves at least two mechanisms. Firstly, a rapid increase of $[Ca^{2+}]_i$ which could be caused by a reduction of myoplasmic Ca^{2+} buffering, reduced sarcoplasmic reticulum Ca^{2+} uptake or by inhibition of the Na^+–Ca^{2+} exchanger. Secondly, a slower increase of $[Ca^{2+}]_i$ is a consquence of enhanced Na^+–H^+ exchange (leading to increased $[Na^+]_i$) and a subsequent Ca^{2+} influx via the Na^+–Ca^{2+} exchanger. Preliminary accounts of some of this work have been reported (Allen, Westerblad & Cairns, 1992).

METHODS

Isolation of ventricular myocytes

Adult Sprague–Dawley rats (200–300 g) were killed by cervical disarticulation. The heart was excised and washed and then perfused retrogradely through the aorta at 10 ml/min with a calcium-free Hepes-buffered solution (50 μM EGTA) at 37 °C. After 5 min the perfusion solution was changed to one containing 25 μM Ca^{2+}, 1 mg/ml collagenase (Worthington CLS II, USA),

0·1 mg/ml protease (Sigma, St Louis, MO, USA) and 50 μM Ca-EGTA. The first 10 ml of this perfusate was discarded and the remaining perfusion solution recirculated for 15 min. Finally, segments of ventricular muscle were removed, chopped into small pieces, and then gently triturated with a plastic pipette to release the cells. The first supernatant contained enzyme and was discarded. The cells were then resuspended in a Hepes-buffered solution (see below) containing 250 μM Ca^{2+} and maintained at room temperature.

Solutions and drugs

The standard solution used was a Hepes-buffered solution of composition (mM): Na^+, 140; K^+, 4; Mg^{2+}, 1·2; Ca^{2+}, 1·0; Cl^-, 137·5; $H_2PO_4^-$, 1·2; SO_4^{2-}, 1·2; glucose, 11; Hepes, 20. Solutions were equilibrated with O_2 and pH was adjusted to 7·4. In addition, all solutions contained 50 μM Ca-EGTA and approximately 0·2 % fetal calf serum which assisted cell survival and viability. Experiments were performed at a room temperature of 24 °C. Lactate was added as sodium lactate without osmotic compensation.

Stimulation and cell length measurements

Healthy cells attached to the base of the chamber and were stimulated to contract by field stimulation (5 ms duration) at a frequency of 0·4 Hz. The extent of cell shortening (percentage change or absolute units (μm)) was determined from cell length measurements. Cells were illuminated using red light and the length was monitored by a video camera and a length analyser.

Loading cells with fluorescent indicators

Cells were loaded with the membrane-permeable acetoxymethyl (AM) ester forms of the fluorescent indicators 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) or fura-2 (obtained from Molecular Probes, USA) to measure the pH_i or the $[Ca^{2+}]_i$, respectively. The indicators were dissolved in dimethyl sulphoxide and cells were loaded using the following protocols: (i) 5 μM BCECF AM for 30 min or (ii) 1–10 μM fura-2 AM for 15 min. Cell loading using these protocols showed relatively homogeneous fluorescence when examined at high power in UV light, suggesting that loading of intracellular organelles was minimal.

Fluorescence measurements

The apparatus and optical arrangements used for the measurement of fluorescent light emission were similar to those described in detail by Lee, Westerblad & Allen (1991).

BCECF. The pH-dependent signal of BCECF was obtained by illuminating at 490 and 435 nm and dividing the emitted light signals (> 520 nm). The background fluorescence, determined by moving the cells out of the field of view, was small (< 5 % total fluorescent signal) and was subtracted from the total signal. The autofluorescence determined by illuminating unloaded cells was also small (< 2 % total signal) and has been ignored. There was interference between fluorescence measurements and the cell shortening signal so cell shortening was measured most of the time and at about 30 s intervals the two fluorescent signals were recorded.

Fura-2. The calcium-dependent signal of fura-2 was obtained by illuminating at 340 and 380 nm, and recording the emitted light at 505 ± 5 nm. The background fluorescence was subtracted. The autofluorescence varied between 4 and 15 % at 340 nm and 1 and 6 % at 380 nm in different experiments. However, these signals did not change during exposure to acidosis and no correction has been made for them except in the experiments with EIPA (ethylisopropylamiloride) in which autofluorescence increased further. When fura-2 was used fluorescent signals and cell shortening were measured simultaneously.

Calibrations of BCECF signals

Calibrations of BCECF signals were performed *in vivo* in the following solution (mM): K^+, 150; Na^+, 5; Cl^-, 165; Hepes, 5; Mes (2-(*N*-morpholino)ethanesulphonic acid), 5; Trizma, 5; in which the pH was changed from 5·0 to 9·0. The intracellular pH was set to the extracellular pH (pH_o) with 10 μM nigericin, a proton and potassium ionophore. The ratios obtained were fitted to the following equation (Grynkiewicz, Poenie & Tsien, 1985):

$$\mathrm{pH} = \mathrm{p}K_a - \log[(R - R_{min})/(R_{max} - R)] - \log(S_{f2}/S_{b2}), \quad (1)$$

where pK_a is −log(dissociation constant); R is the 490/435 ratio; R_{min} and R_{max} are the ratios when the indicator has no protons bound (pH 9·0) or with maximum proton binding (pH 5·0); S_{f2}

and S_{b2} are constants showing the fluorescence intensity during illumination at 435 nm at pH 9·0 and 5·0. The mean values obtained ($n = 8$) were $R_{min} = 1{\cdot}34 \pm 0{\cdot}02$, $R_{max} = 0{\cdot}34 \pm 0{\cdot}01$, $S_{f2}/S_{b2} = 0{\cdot}80 \pm 0{\cdot}02$. The pK_a calculated from our data was 7·18. All BCECF ratios were converted to pH units from this curve.

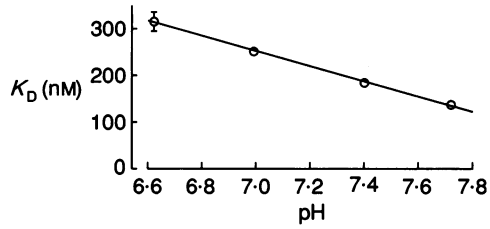

Fig. 1. The pH sensitivity of fura-2. *In vitro* determinations of the K_D of fura-2 for Ca^{2+} plotted against pH. Each point shows the mean and S.E.M. (where bigger than the symbol) of three to five determinations.

Calibration of fura-2 signals

We performed *in vivo* calibrations to determine the relation between the fura-2 ratio and the $[Ca^{2+}]_i$. The Ca^{2+} ionophore, ionomycin (5 μM), was first added so that $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ would equilibrate. Zero $[Ca^{2+}]_o$ (20 mM EGTA) was then applied and the $[Ca^{2+}]_i$ fell slowly to R_{min}. The $[Ca^{2+}]_o$ was then changed to 20 mM to produce R_{max} and this was usually associated with a massive contracture with many cells detaching from the base of the chamber and floating away. The $[Ca^{2+}]$ was then changed to 200 nM using EGTA buffers and in two cells we managed to get stable intermediate ratio values. The $[Ca^{2+}]$ was determined according to the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}] = K_D b(R - R_{min})/(R_{max} - R), \quad (2)$$

where $R_{min} = 0{\cdot}57 \pm 0{\cdot}02$, $R_{max} = 7{\cdot}68 \pm 0{\cdot}72$, and b (= 6·4) is the fluorescence intensity during illumination at 380 nM with 0 Ca^{2+} and 20 mM Ca^{2+}. The K_D (dissociation constant) was determined to be 585 nM by fitting our data for the intermediate ratio where $[Ca^{2+}] = 200$ nM.

Fura-2 was originally described as relatively insensitive to changes of pH (Grynkiewicz *et al.* 1985). However, in agreement with Uto, Arai & Ogawa (1991) we found considerable pH sensitivity. *In vitro* calibration curves were determined for pH values ranging from 6·6 to 7·7 pH units. R_{min} and R_{max} were determined with 0 Ca^{2+} (+20 mM EGTA) and 20 mM Ca^{2+}; intermediate $[Ca^{2+}]$ levels were determined by mixing Ca-EGTA and EGTA in ratios of 1:3, 1:1 and 3:1 and calculating the free $[Ca^{2+}]$ given the known effect of pH on EGTA binding constants (Martell & Smith, 1974). Figure 1 shows the measured K_D values determined from these experiments. The K_D at the normal extracellular pH of 7·4 was 185 nM and the slope of the line in Fig. 2*B* is −160 nM/pH unit. This means that fura-2 is less sensitive to Ca^{2+} as the pH falls and failure to correct for this effect would underestimate the true $[Ca^{2+}]_i$. Therefore the $[Ca^{2+}]_i$ determined during exposure to lactate was corrected for measured changes in pH as determined in BCECF studies.

Statistics

All data are presented as the means ± S.E.M. of n cells. Statistical significance was tested with Student's paired t test and $P < 0{\cdot}05$ taken as significant.

RESULTS

Changes of pH_i and contraction during exposure to lactate

The resting pH_i of ventricular myocytes was $7{\cdot}42 \pm 0{\cdot}03$ pH units ($n = 42$), following equilibration in the Hepes-buffered solution for 10–20 min. An intracellular acidosis was produced by exposing the myocytes to 20 mM lactate at

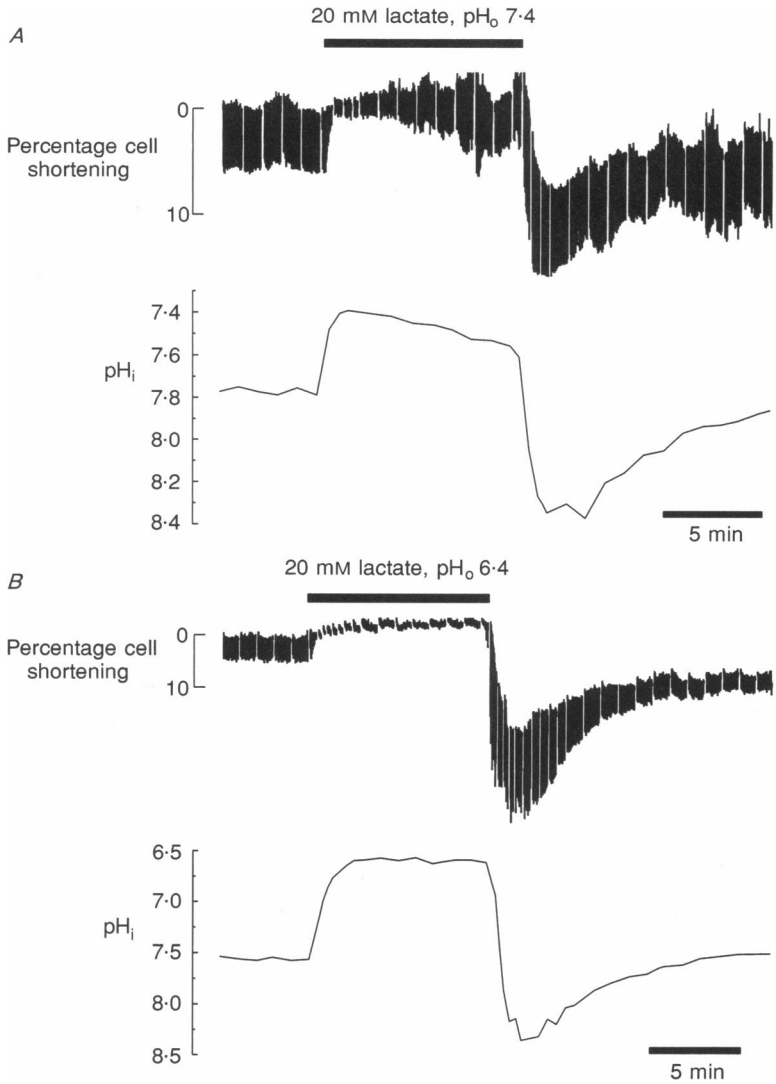

Fig. 2. The effects of 20 mM lactate on cell shortening (upper traces) and pH_i (lower traces). *A*, extracellular pH 7·4; *B*, extracellular pH 6·4. Cell shortening is downwards; individual shortenings are fused together on this time base. The breaks at approximately 30 s intervals are the times at which the fluorescence ratio was determined. Different cells in *A* and *B*. The cell in *A* had an unusually alkaline pH_i but otherwise the record is representative. In *B* shortening was undetectable in lactate; the record shows the noise level of the shortening signal.

the normal extracellular pH (pH_o 7·4) (cf. de Hemptine, Marrannes & Vanheel, 1983). Figure 2*A* shows the effects of 20 mM lactate, applied for 10 min, on the extent of cell shortening and the pH_i in a ventricular myocyte. Initially there was a rapid decline of cell shortening (the first 1–2 min) which showed a close temporal relationship with a decline of pH_i. In seventeen cells, the pH_i fell by 0·24 ± 0·02 pH

units at the time when cell shortening was maximally reduced to 22 ± 4 %. In the subsequent 10 min, during the maintained exposure to lactate, there was a slower partial recovery of both shortening and pH_i. During this period the pH_i recovered by $0{\cdot}16 \pm 0{\cdot}02$ pH units and shortening to 81 ± 6 % of the control length. This recovery of pH_i during the lactate challenge suggests that a proton-regulating process has been active, such as the Na^+–H^+ exchanger.

When lactate was washed out of the bath, the myocyte developed a contracture and the extent of cell shortening increased about twofold (Fig. 2*A*). During early wash-out pH_i reached $7{\cdot}71 \pm 0{\cdot}07$ pH units; thereafter, pH_i and shortening recovered towards the initial control values.

Effects of lactate exposure at reduced pH_o

During myocardial ischaemia the pH_o falls (e.g. Vanheel, de Hemptine & Leusen, 1990) because protons can no longer be removed by the circulation. We attempted to mimic the ischaemic situation with the isolated cells by applying lactate at a reduced extracellular pH (pH_o 6·4). We predicted that with this protocol it would be possible to (i) induce a greater intracellular acidosis since more lactate in the extracellular solution exists in the membrane-permeable uncharged form and (ii) the slow recovery of pH_i, seen during exposure to lactate at pH_o 7·4, might be attenuated since reducing the pH_o inhibits the activity of the Na^+–H^+ exchanger (Lazdunski, Frelin & Vigne, 1985; Vaughan-Jones & Wu, 1990*b*).

Figure 2*B* shows the effects of adding 20 mM lactate at pH_o 6·4 for 10 min on cell shortening and pH_i, in a myocyte. Again there was an initial rapid decline of shortening and pH_i, both of which were greater than at pH_o 7·4. In eight myocytes, the pH_i fell by $0{\cdot}77 \pm 0{\cdot}06$ pH units in the first 2 min, by which time contraction was virtually abolished. In contrast to the effect with lactate at the normal pH_o, the pH_i did not recover in the continued presence of lactate (increase of $0{\cdot}04 \pm 0{\cdot}03$ pH units). Similarly, contraction remained abolished in all but one myocyte which displayed a small partial recovery. This result suggests that the proton regulatory process might well have been impaired by lowering pH_o.

During wash-out of lactate the mycytes went into a severe contracture and many did not recover. The surviving mycoytes displayed considerable twitch potentiation (Fig. 2*B*) and showed a large alkalinization (to $8{\cdot}10 \pm 0{\cdot}15$ pH units).

Effects of Na^+–H^+ exchange inhibitors on changes of pH_i and contraction during exposure to lactate

Inhibitors of the Na^+–H^+ exchanger, amiloride and EIPA (Kleyman & Cragoe, 1988), were used to test whether this cell membrane exchanger was involved in the regulation of pH_i during lactate application. The effect of both 1 mM amiloride or 10 μM EIPA on the resting pH_i was identical; a slowly developing acidosis of $0{\cdot}07 \pm 0{\cdot}01$ pH units ($n = 15$) had occurred after 5 min. This suggests that the Na^+–H^+ exchanger operates to extrude protons at a slow rate under resting conditions at normal extracellular pH (cf. Deitmer & Ellis, 1980).

Figure 3 demonstrates the effects of applying lactate (pH_o 7·4) to the same cell in the absence and presence of 1 mM amiloride. The magnitude of the initial fall of pH_i was similar, although slightly greater during Na^+–H^+ exchange blockade than in

control conditions, i.e. $0{\cdot}29 \pm 0{\cdot}02$ *versus* $0{\cdot}24 \pm 0{\cdot}02$ pH units ($n = 11$). Therefore the initial fall in pH_i must reflect a small component due to rapid pH regulation by the Na^+–H^+ exchanger in addition to buffering (e.g. Vaughan-Jones & Wu, 1990*a*). However, the most striking feature was that amiloride prevented both the normal

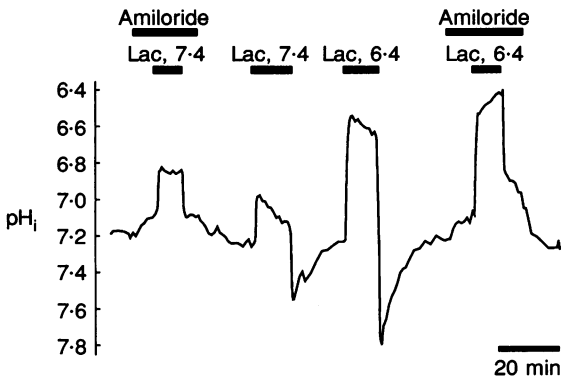

Fig. 3. Effect of amiloride (1 mM), an inhibitor of Na^+–H^+ exchange, on the pH_i changes in response to 20 mM lactate (Lac) at various pH_o values.

recovery of pH_i during maintained exposure to lactate and the alkalinization during wash-out. Identical responses were obtained in the presence of EIPA. The pH_i remained unchanged in the continued presence of lactate and Na^+–H^+ exchange blockers and returned to control on wash-out during Na^+–H^+ exchange blockade. This indicates that both the recovery of pH_i and the alkalinization involve the Na^+–H^+ exchange.

When lactate was added during Na^+–H^+ exchange blockade, cell shortening was at first reduced to 26 ± 6 % of the control, which is similar to that observed when the exchanger is active, but the subsequent recovery was attenuated, reaching only 40 ± 11 % ($n = 8$) of the control value compared with 80 % when the exchanger is active. In addition, wash-out of lactate in the presence of inhibitor resulted in a much reduced contracture and reduced twitch potentiation (see Fig. 6).

The lack of recovery of pH_i during exposure to lactate at pH_o 6·4 suggests that the Na^+–H^+ exchanger might be inactive under these conditions (cf. Vanheel *et al.* 1990). Figure 3 shows that this is not the case since the addition of lactate in the presence of the Na^+–H^+ exchange inhibitor caused an additional slowly developing acidosis compared with effects in the absence of the inhibitor. In five experiments when lactate was applied during Na^+–H^+ exchange blockade the pH_i fell rapidly by $0{\cdot}65 \pm 0{\cdot}05$ pH units but showed a further decline of $0{\cdot}15 \pm 0{\cdot}03$ pH units over 10 min of lactate exposure. This implies that the Na^+–H^+ exchanger must normally have been active when lactate was applied at the reduced pH_o in order to maintain pH_i and prevent the further decline.

The mechanism for the slowly developing acidosis after the initial rapid fall, when lactate was added in the presence of Na^+–H^+ exchange inhibitors, is unclear. In order to determine the effect of reducing pH_o alone, we reduced pH_o to 6·4 with HCl (not shown). This procedure caused a large, slowly developing acidosis

(cf. Deitmer & Ellis, 1980). This result suggests that the slowly developing component of the acidosis observed after application of lactate at pH_o 6·4 in the presence of amiloride is due to the entry of protons in the ionized form though other explanations are not excluded (cf. Vaughan-Jones & Wu, 1990*b*).

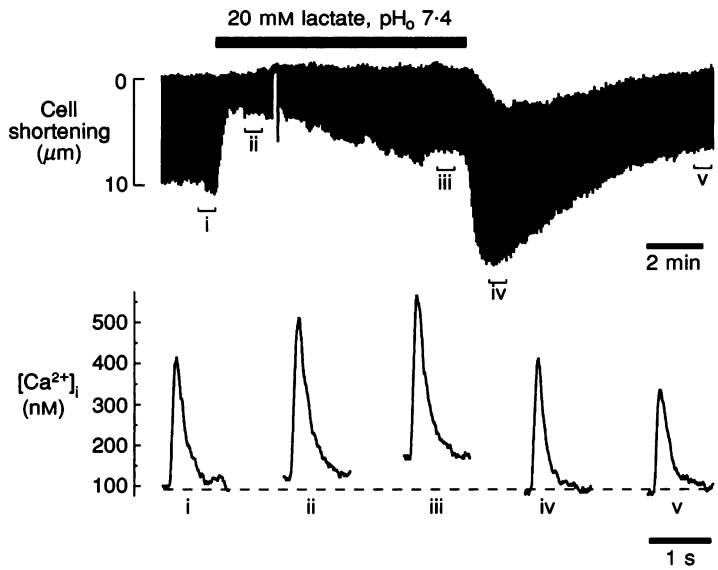

Fig. 4. Effects of 20 mM lactate (pH_o = 7·4) on cell shortening (*A*) and Ca^{2+} transients (*B*). Ca^{2+} transients were averaged from eight contractions at the times indicated in *A*. In calibrating the ratio signals correction has been made for the effects of pH_i on fura-2 (see Methods). The short break in *A* was caused by the stimulus strength falling below threshold.

Changes of $[Ca^{2+}]_i$ *and contractions during exposure to lactate*

In thirty-eight myocytes, the average diastolic and systolic $[Ca^{2+}]_i$ values were 101 ± 9 and 394 ± 30 nM, respectively ($[Ca^{2+}]_o$ = 1 mM, 0·4 Hz stimulation frequency). When $[Ca^{2+}]_o$ was increased to 2 or 3 mM this resulted in higher $[Ca^{2+}]_i$. For example, with 2 mM $[Ca^{2+}]_o$ ($n = 6$), the diastolic $[Ca^{2+}]_i$ increased to 135 ± 12 nM and the systolic $[Ca^{2+}]_i$ to 605 ± 38 nM and the cell shortening increased about twofold.

The changes of $[Ca^{2+}]_i$ resulting from exposure to lactate at pH_o 7·4 are shown in Fig. 4. In the first 1–2 min after applying 20 mM lactate, when shortening was maximally reduced (and pH_i had fallen by about 0·25 pH units), the diastolic and systolic $[Ca^{2+}]_i$ and amplitude of the Ca^{2+} transient had increased. On average, in eight cells, the diastolic $[Ca^{2+}]_i$ increased by 38 ± 7 nM and systolic $[Ca^{2+}]_i$ increased by 95 ± 24 nM. This corresponded to an 18 ± 6 % increase in the amplitude of the Ca^{2+} transient. As the period of exposure to lactate continued, there was a further progressive rise in the $[Ca^{2+}]_i$ and twitch shortening partially recovered. Immediately prior to wash-out the average diastolic $[Ca^{2+}]_i$ had increased by a further 41 ± 12 nM, the systolic $[Ca^{2+}]_i$ by a further 141 ± 42 nM, and the Ca^{2+}

transient had increased by 49 ± 13 % ($n = 8$) of the initial control. When lactate was washed out of the bath a contracture developed and twitch shortening was potentiated while the diastolic and systolic $[Ca^{2+}]_i$ were not significantly different from the initial controls.

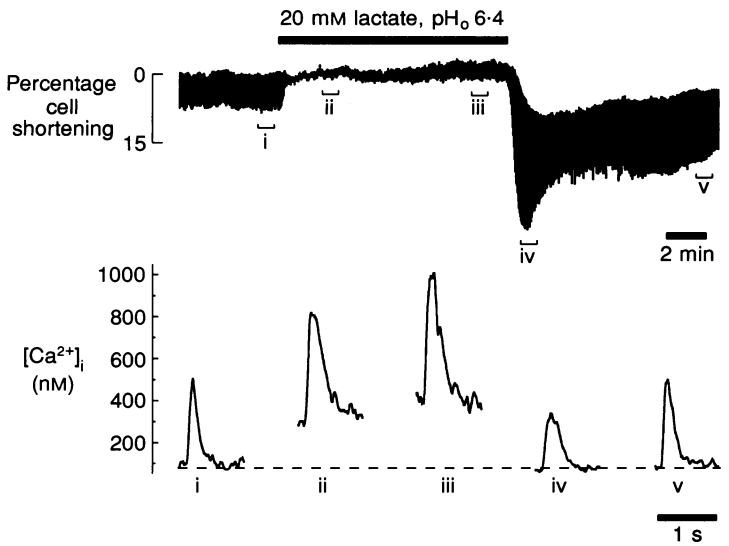

Fig. 5. Effects of 20 mM lactate ($pH_o = 6·4$) on cell shortening (*A*) and Ca^{2+} transients (*B*). Note the prolongation of the time course of the Ca^{2+} transients in the presence of lactate. pH_o was 7·4 during remainder of the record.

Effects of lactate exposure on $[Ca^{2+}]_i$ at reduced extracellular pH

Under these conditions, which more closely resemble ischaemia, the changes in $[Ca^{2+}]_i$ are similar to those at the normal pH_o but are larger (Fig. 5). Soon after lactate was applied the diastolic $[Ca^{2+}]_i$ had increased by 74 ± 8 nM and the systolic $[Ca^{2+}]_i$ increased by 128 ± 35 nM ($n = 6$). In the remaining period of the lactate challenge the $[Ca^{2+}]_i$ further increased so that immediately prior to wash-out of lactate the diastolic and systolic $[Ca^{2+}]_i$ values had increased by a further 48 ± 15 and 323 ± 71 nM; the Ca^{2+} transient had increased by 129 ± 30 % of the initial control value. These large increases in $[Ca^{2+}]_i$ were associated with either no recovery or very little recovery of cell shortening. A likely explanation for the lack of recovery of shortening is that the decline in Ca^{2+} sensitivity due to the acidification (Fabiato & Fabiato, 1978) is so great that the systolic $[Ca^{2+}]_i$ is lower than that required to initiate contraction. When lactate was removed a huge contracture and potentiation of twitches developed and was associated with a smaller Ca^{2+} transient (Fig. 5). The diastolic $[Ca^{2+}]_i$ fell to below the initial control and the Ca^{2+} transient was reduced to 80 ± 15 % of the initial control.

Effects of Na^+–H^+ exchange blockade on changes of $[Ca^{2+}]_i$ during exposure to lactate

Our earlier pH_i studies suggested that the Na^+–H^+ exchanger was active in extruding protons under resting conditions and during exposure to lactate. Stimulation of the Na^+–H^+ exchanger would be expected to increase the $[Na^+]_i$ (for

references see Introduction) which would, subsequently, bias the Na^+–Ca^{2+} exchanger, resulting in a net Ca^{2+} influx. This effect could account for the slow increases in $[Ca^{2+}]_i$. These processes would directly increase the diastolic $[Ca^{2+}]_i$, which would cause greater Ca^{2+} loading of the sarcoplasmic reticulum and thereby

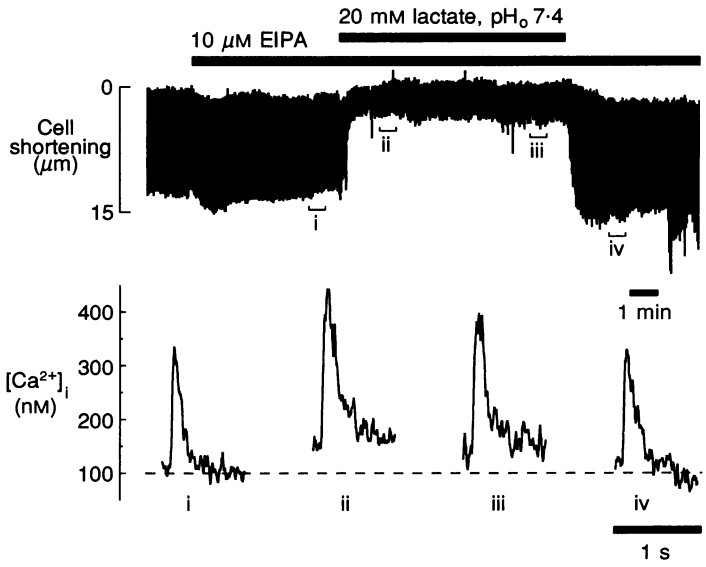

Fig. 6. Effect of EIPA (10 μM), an inhibitor of Na^+–H^+ exchange, on cell shortening (*A*) and Ca^{2+} transients (*B*) during 20 mM lactate exposure ($pH_o = 7·4$). Note that towards the end of the record irregular contractions started to occur.

produce a larger Ca^{2+} transient. To test this hypothesis we measured $[Ca^{2+}]_i$ during exposure to lactate in the presence of Na^+–H^+ exchange inhibitors. Amiloride (1 mM) caused a huge increase of fluorescence in cells and hence was unsuitable for these experiments. Instead, we used EIPA (10 μM) which caused only a small increase in fluorescence which was corrected for (see Methods).

Figure 6 shows the changes in $[Ca^{2+}]_i$ which occur during lactate exposure in the presence of EIPA. Clearly EIPA did not prevent the early lactate-induced changes in diastolic or systolic $[Ca^{2+}]_i$, nor the early increase of the amplitude of the Ca^{2+} transient. During the early acidosis ($n = 4$), the diastolic $[Ca^{2+}]_i$ increased by 49 ± 9 nM, the systolic $[Ca^{2+}]_i$ by 165 ± 75 nM and the amplitude of the Ca^{2+} transient by 38 ± 3 % of the initial control values in EIPA. The important feature to note is that the slower rise in $[Ca^{2+}]_i$ was prevented by exposure to EIPA. In this situation, none of the diastolic $[Ca^{2+}]_i$, the systolic $[Ca^{2+}]_i$ or the Ca^{2+} transient changed significantly after the initial rapid increase. Following wash-out of lactate the myoplasmic $[Ca^{2+}]_i$ returned towards the initial control levels in EIPA.

We can thus show at least two components to the acidosis-induced rise in $[Ca^{2+}]_i$: (i) a rapid component which is insensitive to Na^+–H^+ exchange inhibition and (ii) a more slowly developing component which is eliminated by Na^+–H^+ exchange inhibitors.

DISCUSSION

Regulation of pH_i during lactate exposure

The slow recovery of pH_i during lactate exposure at the normal pH_o is likely to be due to proton extrusion by the Na^+–H^+ exchanger since it was abolished with Na^+–H^+ exchange inhibitors. This interpretation is also supported by the studies which show a rise in $[Na^+]_i$ during acidosis (for references see Introduction). During prolonged exposure to lactate at the reduced pH_o the pH_i did not recover but, nevertheless, the Na^+–H^+ exchanger was still active since an additional acidosis occurred over this period in the presence of amiloride. The difference must therefore represent extrusion of protons by the Na^+–H^+ exchanger. At normal pH_o the amiloride-sensitive component of pH_i recovery averaged 0·16 pH units over 10 min. The intrinisic buffering power at this pH_i is 18 mM/pH unit (Vaughan-Jones & Wu, 1990*a*) and to this must be added the buffering due to intracellular lactate (2·3 × intracellular lactate concentration; Roos & Boron, 1981). Intracellular lactate was calculated to be 14 mM, giving a total buffer power of 50 mM/pH unit. Thus the calculated proton efflux rate on the exchanger is 0·8 mM protons/min under these conditions. At pH_o 6·4, the amiloride-sensitive component of pH change averaged 0·19 pH units/10 min (the sum of the pH_i change in the absence of amiloride, 0·04, and the pH_i change in its presence, 0·15); the total buffering power at the relevant pH_i including intracellular lactate is 104 mM/pH unit so that the calculated proton efflux on the exchanger is 2·0 mM protons/min. Thus the proton extrusion rate appears to be more than twice as large under the more acid conditions, suggesting that activation of the exchanger by intracellular acidosis is greater than the inhibition by extracellular acidosis. This is very different from the results of Vanheel *et al.* (1990) who found that Na^+–H^+ exchange activity (measured by the increase in $[Na^+]_i$) was completely prevented by decreasing pH_o from 7·5 to 7·0 and suggested therefore that Na^+–H^+ exchange would not operate under ischaemic conditions. However, our result is close to the predictions of data of Vaughan-Jones and colleagues. They showed that a reduction in pH_o from 7·4 to 6·4 caused approximately a 5-fold fall in proton efflux (Vaughan-Jones & Wu, 1990*b*) while a decrease in pH_i from 7·15 to 6·68 caused approximately a 5- to 10-fold increase in proton efflux (Kaila & Vaughan-Jones, 1987).

Regulation of $[Ca^{2+}]_i$ during lactate exposure

The lactate-induced intracellular acidosis caused an increase in the diastolic and systolic $[Ca^{2+}]_i$, and amplitude of the Ca^{2+} transient which was comparable to that observed in ischaemia (e.g. Allen *et al.* 1989). Since fura-2 was found to be sensitive to changes of pH it was necessary to correct the $[Ca^{2+}]_i$ using the changes of pH_i measured during lactate exposure. The correction factor determined from *in vitro* calibration was assumed to be the same as the *in vivo* situation, although this may not be the case. The early rapid increase in $[Ca^{2+}]_i$ was not detected without this correction for pH though an early rapid increase in $[Ca^{2+}]_i$ has been observed with indo (Kohmoto *et al.* 1990) which is less sensitive to pH (Westerblad & Allen, 1993).

A number of mechanisms exist which may contribute to the rise in $[Ca^{2+}]_i$ during an intracellular acidosis (for review see Orchard & Kentish, 1990). Our finding that

EIPA prevented the gradual increases in $[Ca^{2+}]_i$ confirms the work of Kim & Smith (1988) and Harrison *et al.* (1992). Therefore the slow component of the rise in $[Ca^{2+}]_i$ required stimulation of the Na^+–H^+ exchanger. The elevation of $[Na^+]_i$ resulting from enhanced Na^+–H^+ exchange would change the driving force on the Na^+–Ca^{2+} exchanger to favour a net Ca^{2+} influx. This would explain the gradual rise in diastolic $[Ca^{2+}]_i$ and subsequent loading of the sarcoplasmic reticulum can explain the increased Ca^{2+} transients.

There are several possible explanations for the early increase in $[Ca^{2+}]_i$ which occurs independently of Na^+–H^+ exchange. Firstly, reduced Ca^{2+} buffering by myoplasmic Ca^{2+} binding proteins may have a role (Bers & Ellis, 1982; Vaughan-Jones *et al.* 1983). For instance, the binding of Ca^{2+} to troponin-C is reduced under acid conditions (Blanchard & Solaro, 1984). However, there is relatively little Ca^{2+} bound to troponin-C at rest so that the Ca^{2+} must be displaced from other myoplasmic binding sites if this mechanism is responsible for the early rise in diastolic $[Ca^{2+}]_i$. Secondly, a proton-induced slowing of Ca^{2+} uptake by the sarcoplasmic reticulum (Mandel *et al.* 1982) could increase the systolic $[Ca^{2+}]_i$ which would also be consistent with the prolonged Ca^{2+} transient. However, either of these two mechanisms ought to give only a transient rise in diastolic $[Ca^{2+}]_i$ since in the steady state the diastolic $[Ca^{2+}]_i$ depends only on the balance of inward leaks and outward pumps across the surface membrane. Thirdly, protons may directly inhibit the Na^+–Ca^{2+} exchanger (Earm & Irisawa, 1986) and since Ca^{2+} is normally extruded by the Na^+–Ca^{2+} exchanger in resting muscle (for review see Eisner & Lederer, 1989) this would directly increase diastolic $[Ca^{2+}]_i$ and subsequently increase the amplitude of the Ca^{2+} transient.

Relevance of the present results to ischaemia

In our earlier study of simulated ischaemia (Allen *et al.* 1989) we found a rise in $[Ca^{2+}]_i$ which was comparable in magnitude to that produced by application of lactate and in both ischaemia and acidosis the Ca^{2+} transients were prolonged. In addition glycogen depletion, which reduces the acidosis, also prevents much of the rise of $[Ca^{2+}]_i$. It seems clear, therefore, that acidosis due to lactic acid accumulation contributes to the rise of $[Ca^{2+}]_i$ during ischaemia but two further questions arise: (i) do other cellular changes during ischaemia contribute to the rise in $[Ca^{2+}]_i$ and (ii) what is the mechanism whereby acidosis causes increased $[Ca^{2+}]_i$? (i) It is already clear that other processes contribute to the rise of $[Ca^{2+}]_i$. Mohabir, Lee, Kurz & Clusin (1991) have shown that there is a rise in $[Ca^{2+}]_i$ after only 1–2 min of ischaemia which is not associated with an appropriate acidosis but they concluded that the larger rise in $[Ca^{2+}]_i$ after 5–15 min was caused by acidosis. The possibility that inhibition of the Na^+ pump is involved, leading to increased $[Na^+]_i$ and hence $[Ca^{2+}]_i$, has often been suggested but experimental tests have not supported this hypothesis, at least in early ischaemia (Kléber, 1983). It is also likely that changes in the action potential which become prominent after 10–15 min are involved in the subsequent decline in Ca^{2+} transients which occurs later in ischaemia (Allen *et al.* 1989). (ii) A number of studies have suggested that operation of the Na^+–H^+ exchanger leading to Na^+ and Ca^{2+} loading is the mechanism by which acidosis causes increased $[Ca^{2+}]_i$ during ischaemia (Tani & Neely, 1989; Pike, Kitakaze & Marban, 1990). This

hypothesis has received strong support from the experiments of Murphy, Perlman, London & Steenbergen (1991), who showed that the rise in both $[Na^+]_i$ and $[Ca^{2+}]_i$ are inhibited by pretreatment with amiloride. On the other hand, there are a number of studies which have failed to show increases in $[Na^+]_i$ during ischaemia (Kléber, 1983; Vanheel *et al.* 1990) and, as noted above, Vanheel *et al.* suggested that extracellular acidosis was inhibiting the exchanger. This was also the basis for the hypothesis of Lazdunski *et al.* (1985) in which it was proposed that reactivation of the Na^+–H^+ exchanger on reperfusion led to Na^+ and therefore Ca^{2+} loading. As noted above, our results show that the Na^+–H^+ exchanger operated at an *increased* rate when both the pH_i and the pH_o were similar to those during ischaemia. However, our finding of a rapid increase in $[Ca^{2+}]_i$ even when the Na^+–H^+ exchanger was inhibited suggests an additional mechanism whereby acidosis can elevate $[Ca^{2+}]_i$.

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