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THE EFFECTS OF METABOLIC INHIBITION ON INTRACELLULAR CALCIUM AND pH IN ISOLATED RAT VENTRICULAR CELLS

BY D. A. EISNER, C. G. NICHOLS*, S. C. O'NEILL, G. L. SMITH AND M. VALDEOLMILLOS

From the Department of Physiology, University College London, Gower Street, London WC1E 6BT

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SUMMARY

1. Intracellular calcium concentration ($[Ca^{2+}]_i$) and pH (pH_i) were measured in single, isolated rat ventricular myocytes using, respectively, the fluorescent indicators Fura-2 and BCECF (2′,7′-bis(carboxyethyl-5(6)-carboxyfluorescein). Contraction was measured simultaneously. The intracellular calibration of BCECF is demonstrated. In a HEPES-buffered bathing solution of pH 7·4, pH_i had a mean value of $7{\cdot}16 \pm 0{\cdot}05$ (mean ± S.E.M.).

2. Addition of NH_4Cl (5–20 mM) produced an intracellular alkalosis that was associated with an increase of contraction amplitude. Removal of NH_4Cl produced an acidosis and decrease of contraction.

3. The addition of 2 mM-cyanide (CN^-) to inhibit oxidative phosphorylation had variable effects on contraction amplitude. Changes of contraction amplitude could largely be accounted for by changes in the systolic Ca^{2+} transient.

4. CN^- addition increased lactic acid production. However, in the majority of experiments, this was not accompanied by an intracellular acidosis.

5. Anaerobic glycolysis was inhibited by either removal of glucose, addition of deoxyglucose, or addition of iodoacetate. Under these conditions the application of CN^- decreased systolic $[Ca^{2+}]_i$ and contraction amplitude. This was sometimes preceded by a transient *increase* of systolic $[Ca^{2+}]_i$ and contraction amplitude.

6. When glycolysis was inhibited, the subsequent addition of CN^- always increased diastolic $[Ca^{2+}]_i$ and produced a contracture. The increase of $[Ca^{2+}]_i$ occurred *before* the contracture. However, once the contracture had developed, decreasing $[Ca^{2+}]_i$ (by removal of external Ca^{2+}) did not cause relaxation.

7. With glycolysis inhibited, addition of CN^- resulted in a large ($0{\cdot}51 \pm 0{\cdot}05$ pH unit) acidosis that was sometimes preceded by an alkalosis. This acidosis was unaffected by removal of external Ca^{2+} or external alkalinization. Calculations show that some of this acidosis may result from protons released by ATP hydrolysis.

8. If the acidosis produced by metabolic blockade was partly reversed by adding NH_4Cl then a contracture immediately developed. This suggests that the acidosis delays the onset of the contracture.

* Present address: Department of Physiology, University of Maryland School of Medicine, 660 W. Redwood Street, Baltimore, MD 21201, USA.
Authors' names are in alphabetical order.

9. We conclude that metabolic inhibition increases diastolic $[Ca^{2+}]_i$. The accompanying acidosis prevents contraction. Once the contracture has developed it is maintained by factors other than increased $[Ca^{2+}]_i$, possibly by a fall of [ATP].

INTRODUCTION

When cardiac muscle is made ischaemic there is a precipitous decline in the force of contraction. This is accompanied by the inhibition of aerobic metabolism, due to removal of oxygen supply. The metabolic demand is initially compensated for by increased anaerobic glycolysis resulting in lactic acid production. Eventually, however, the rate of glycolysis decreases due to accumulation of products such as lactic acid and depletion of endogenous glycogen (see Allen & Orchard, 1987, for recent references).

Given this background it is not surprising that much work has been done to measure the intracellular concentrations of various ions and metabolites when either aerobic or anaerobic metabolism is inhibited and to relate them to contraction. It is found that inhibition of aerobic metabolism alone depresses contraction. One study has found that this fall of contraction is not accompanied by any change of the Ca^{2+} transient (Allen & Orchard, 1983) whereas another (MacKinnon, Gwathmey & Morgan, 1987) finds a fall of the Ca^{2+} transient, albeit insufficient to account for all the decrease of contraction. The $[Ca^{2+}]_i$-independent decrease of contraction may be attributable to an intracellular acidosis and, particularly, to an increase of intracellular inorganic phosphate (P_i) concentration, (Allen, Morris, Orchard & Pirolo, 1985; Eisner, Elliott & Smith, 1987*a*).

Combined inhibition of aerobic and anaerobic metabolism leads to a decrease of systolic $[Ca^{2+}]_i$ and, eventually, to an increase of diastolic $[Ca^{2+}]_i$ (Cobbold & Bourne, 1984; Smith & Allen, 1988). In addition there is an increase of resting tension or 'contracture' (Allen & Orchard, 1983). The development of this contracture has been reported to precede the increase of diastolic $[Ca^{2+}]_i$ (Allen, Eisner & Orchard, 1984; Eisner, Orchard & Allen, 1984; Cobbold & Bourne, 1984; Smith & Allen, 1988). The contracture has therefore been attributed to a fall of [ATP] producing rigor bridges rather than to an increase of $[Ca^{2+}]_i$. Indeed, according to this hypothesis, the rise of $[Ca^{2+}]_i$ is a secondary *consequence* of a precipitous fall in $[ATP]_i$ (due to enhanced cross-bridge cycling) rather than the cause of the enhanced mechanical activity. The literature is not, however, consistent. While Kim & Smith (1988) found no effect of metabolic blockade on $[Ca^{2+}]_i$, Barry, Peeters, Rasmussen & Cunningham (1987) have reported that an increase in diastolic $[Ca^{2+}]_i$ *precedes* the contraction.

Inhibition of aerobic metabolism should stimulate anaerobic glycolysis and the production of lactic acid which would cause an acidosis. In agreement with this, Allen *et al.* (1985) found in whole ferret hearts that addition of CN^- caused an intracellular acidosis which was not observed after inhibition of glycolysis. In contrast, in ferret papillary muscles, glycolytic inhibition had no effect on the CN^--induced acidosis (Ellis & Noireaud, 1987).

There are three limitations to the work described above. (i) With the exception of the $[Ca^{2+}]_i$ measurements of Cobbold & Bourne (1984) it was performed in multicellular cardiac preparations. Not only may this complicate interpretation due to

accumulation of substances in the restricted extracellular spaces but it means that contraction is measured from more cells than are pH_i and $[Ca^{2+}]_i$. This can cause problems of interpretation when (as may be the case during metabolic inhibition) $[Ca^{2+}]_i$ is non-uniform between different cells (Smith & Allen, 1988). (ii) Many of the measurements of $[Ca^{2+}]_i$ were made using aequorin which is not very sensitive to $[Ca^{2+}]_i$ at normal intracellular resting levels. (iii) $[Ca^{2+}]_i$ and pH_i were not measured under comparable conditions. This is a particularly important problem since, not only do the concentrations of these ions affect each other (Bers & Ellis, 1982; Vaughan-Jones, Lederer & Eisner, 1983) but pH_i and $[Ca^{2+}]_i$ interact to affect contraction (Fabiato & Fabiato, 1978).

Therefore, in this study we have measured $[Ca^{2+}]_i$ and pH_i in isolated myocytes exposed to a few defined conditions of metabolic blockade. The results show striking differences from previous studies in the relationship between $[Ca^{2+}]_i$ and contracture and in the role of pH_i. Some of this work has appeared in abstract form (Eisner, Lederer, Nichols, O'Neill, Smith & Valdeolmillos, 1987*b*; Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1988*a*, *b*).

METHODS

Apparatus. The experiments were performed on single rat ventricular myocytes using a modified fluorescence microscope. Since some of the details of the apparatus are novel it will be described in some detail. A schematic diagram of the set-up is shown in Fig. 1. The cells were placed on a bath (volume 100 μl) with a cover-slip base placed on the stage of an inverting microscope (Nikon Diaphot). Solution was continually superfused over the cells at a rate of about 1 ml min^{-1}. The microscope optics and all objectives transmitted UV light at 340 nm and longer wavelengths. In early experiments a Nikon dry 40 × UV-F objective (NA 0·85) was used although in later experiments a 40 × glycerol immersion (NA 1·3) was used instead. External field stimulation was used to make the cells contract. A near infra-red filter (800 nm, in fact a K filter from a flame photometer) was put in front of the microscope illuminator so that direct light from the microscope lamp would not interfere with the detection of fluorescent light. A 150 W xenon lamp provided the fluorescent illumination. In some early experiments interference filters placed between the light and the microscope were changed manually to select the desired exciting light. However in more recent experiments the filters were in a spinning wheel (Cairn Research, Sittingbourne, Kent) which rotated at 160 Hz. Up to six filters could be in the wheel at any one time. All filters had half-bandwidths of 10 nm. After passing the filters the exciting light was reflected by a dichroic mirror. This had a half-pass wavelength of 400 nm for Fura-2 and of 510 nm for BCECF (2′,7′-bis(carboxyethyl-5(6)-carboxyfluorescein). The reflected light was focused onto the preparation by the objective. The longer wavelength light emitted by the fluorescent indicators was transmitted by the dichroic mirror. It then passed through an adjustable diaphragm that was positioned to ensure that light was only measured from the region occupied by the cell of interest, thus decreasing the background signal. The light then passed through a short pass filter (half-pass wavelength 700 nm) mounted at 45 deg. This allowed the light emitted by the fluorescent indicators to go to the photomultiplier and ensured that longer wavelength light from the microscope illuminator (see above) did not reach the photomultiplier but, instead, was directed to the video camera.

The use of the spinning wheel allows simultaneous measurements at several excitation wavelengths. This has the advantage that it allows one to correct for loss or bleaching of the indicator. However, because each filter is only in the light path for about one-twelfth of the time, the intensity of the fluorescence signals is less than would otherwise be obtained. Therefore, in some experiments when it was important to obtain Ca^{2+} transients with reasonably good signal-to-noise ratios, the wheel was stopped and only one (usually 380 nm) filter was used.

Use of Fura-2 to measure $[Ca^{2+}]_i$. Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) was incorporated into the cells as the acetoxymethyl (AM) ester. A 1 mM-stock solution in DMSO (dimethyl sul-

phoxide) was added to a suspension of cells (about 10^5 cells ml^{-1}) to give a final concentration of 5 μM and incubated at 37 °C for 10 min. After placing some of these loaded cells in the bath and allowing them to settle, the solution flow was started and a cell that responded to stimulation identified. In experiments using the spinning wheel the following filters were used: 340 nm, at which an increase of $[Ca^{2+}]_i$ increases fluorescence; 360 nm, at which it has no effect; and 380 nm, at which it decreases fluorescence. Fluorescence was measured with a 500 nm (half-bandwidth 20 nm) filter. A typical record is shown in Fig. 2*A*. Contraction is accompanied by an increase of

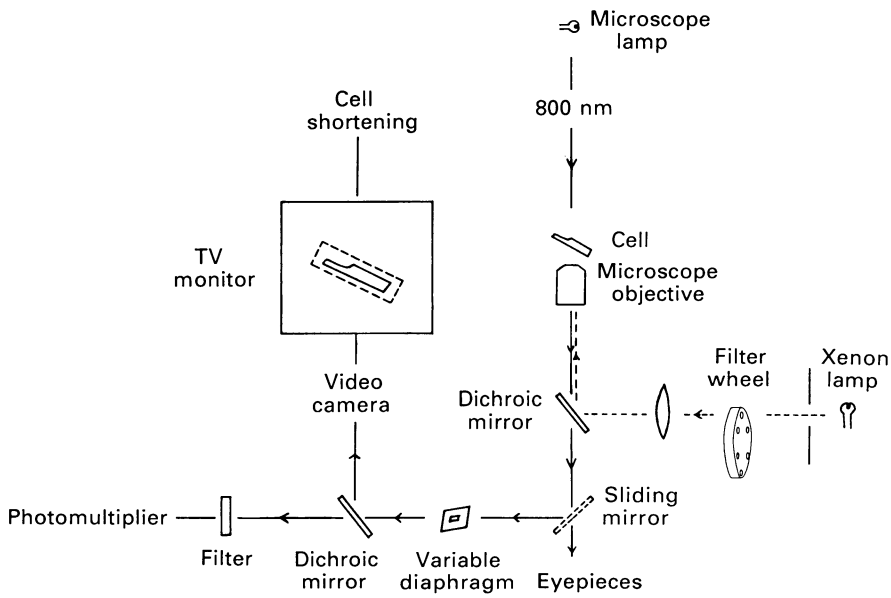

Fig. 1. Diagram of the experimental apparatus. The various components are described in the text. Note in particular the long wavelength (800 nm) filter in front of the microscope lamp and the use of the dichroic mirror to send this long wavelength light to the TV camera rather than to the photomultiplier.

fluorescence at 340, a decrease at 380 and no change at 360 nm. An occasional problem with these measurements is shown in Fig. 2*B*. The 340 nm trace shows a small bump (arrowed) on the descending portion. Similarly a bump can also be seen on the otherwise flat 360 nm trace. These coincide with contraction and are probably artifacts caused by movement of the cell. The example shown is a worst case. Nevertheless, taking the ratio of the signals removes the problem. The ratio was obtained on-line using an analog divide circuit.

Control experiments showed that the intrinsic autofluorescence of the cell was low ($< 2\%$) compared to the Fura-2 signals measured here.

Calibration of Fura-2. It is difficult to calibrate the fluorescence changes produced by Fura-2 added as the AM ester in cardiac muscle. This arises for the following reasons. (i) Some of the AM ester may be only partly hydrolysed in the cytoplasm thus resulting in a component of Ca^{2+}-insensitive fluorescence which will confound attempts to quantify the fluorescence signals in terms of $[Ca^{2+}]_i$ (Highsmith, Bloebaum & Snowdowne, 1986). This does not, however, preclude the use of Fura-2 as a qualitative indicator of changes of $[Ca^{2+}]_i$. (ii) Some of the AM ester may enter intracellular organelles and then be converted to the free indicator. For example it is known that Fura-2-AM can be loaded into isolated mitochondria (Lukacs & Kapus, 1987). In this case part of the observed signal would come from changes of organelle $[Ca^{2+}]$. We have attempted to investigate how much of the Fura-2 is in the cytoplasm by making the surface membrane leaky using saponin or digitonin. Figure 3 shows the effects on fluorescence of adding saponin at a concentration of 50 $\mu g\ ml^{-1}$. This has been shown in isolated rat cardiac muscle to make the cell membrane

leaky to large molecular weight substances without affecting the sarcoplasmic reticular membrane (Kitazawa, 1984). The addition of saponin released the Fura-2 as evidenced by the decrease of fluorescence at 360 nm (the isosbestic wavelength). The kinetics of this release show, however, two components. The first can be approximated to an exponential (time constant 7·5 s). This is then followed by a rather slower phase. In four experiments an average of 71 % of the release occurs in the faster phase. Assuming that saponin only releases cytoplasmic Fura-2, this result would suggest that some of the Fura-2 in the cytoplasm is not freely diffusible and may be bound. The fact that all the signal is eventually released does, however, argue that the Fura-2 in the cell is all in the cytoplasm rather than in intracellular organelles. Similarly two phases of indicator release were observed when Triton X-100 (1 %) was used instead of saponin (not shown). We conclude that the bulk of the Fura-2 signal does come from the cytoplasm.

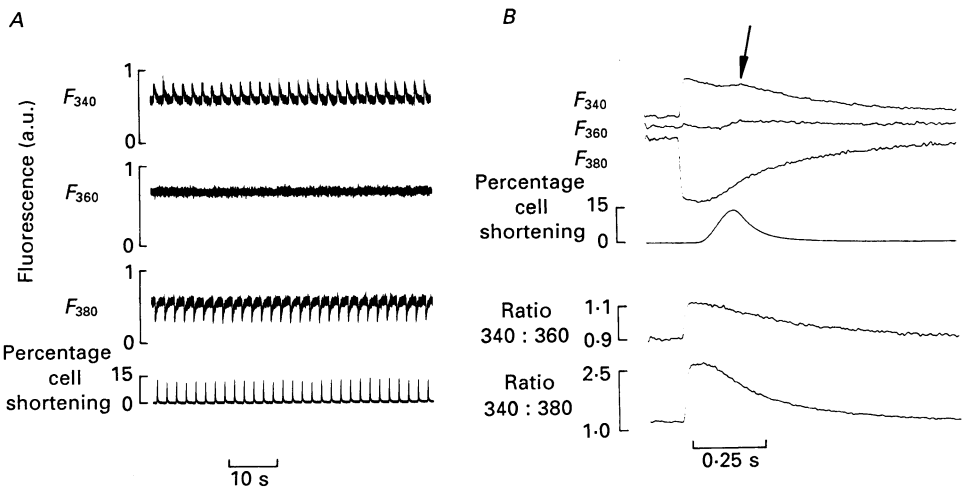

Fig. 2. Ca^{2+} transients recorded with Fura-2. *A*, specimen transients. The top three traces show fluorescence (*F*) in arbitrary units (a.u.) excited at (from top to bottom) 340, 360 and 380 nm. The bottom trace shows contraction. The myocyte was stimulated at 0·5 Hz. *B*, averaged ($n = 16$) records. The top four traces show averaged records of the data from *A*. The lower two records show the ratio of the signal excited at 340 to that excited at 360 nm and the ratio of 340 to 380 nm excitation.

Quantitative calibration of the signals recorded after loading with Fura-2-AM is more difficult. We initially tried an *in vitro* calibration in which the fluorescence ratios are compared with those obtained in calibrating solutions of known $[Ca^{2+}]$. The minimum and maximum ratios were obtained using respectively 0 Ca^{2+} (10 mM-EGTA) or 1 mM-Ca^{2+}. The value of $[Ca^{2+}]_i$ corresponding to any ratio (R) measured in the cell can then be calculated using an equation similar to that given below for BCECF. Unfortunately we found that the ratios measured in the cell often corresponded to impossibly low and sometimes negative values of $[Ca^{2+}]_i$. We attribute this to the problems discussed above and have chosen not to quantify the results.

Use of Fura-2 free acid. In order to avoid the problems of compartmentalization and incomplete hydrolysis mentioned above we have also used cells loaded with the membrane-impermeant free acid form of Fura-2. In these experiments the dye was introduced into the cell via a patch pipette. Pipettes of resistance 2–5 MΩ were filled with the following solution (mM): potassium glutamate, 140; KCl, 10; NaCl, 7; $MgCl_2$, 5; HEPES, 3; Fura-2, 0·1; pH 7·2. After formation of a 'gigaseal' the membrane was ruptured and the Fura-2 allowed to diffuse into the cell. The Fura-2 signals recorded from these cells were calibrated using the above method.

Use of BCECF to measure pH_i. BCECF (Rink, Tsien & Pozzan, 1982) was also added as the AM ester. The cells were incubated in a suspension of 5 μM-BCECF-AM for 30 min. In previous work using BCECF, fluorescence has generally been excited at 490–500 nm at which an alkalosis increases fluorescence and 440 nm at which changes of pH have no effect (Paradiso, Tsien &

Machen, 1987). The *in vivo* excitation spectrum of BCECF is shown in Fig. 4*A*. This was obtained in a single myocyte and shows the increase of fluorescence with alkalosis. It also shows that, at wavelengths below 440 nm, an alkalosis decreases fluorescence. Although the absolute levels of fluorescence are much less than those at longer wavelengths, the fractional change produced by changes of pH is still significant. We have therefore often used 430 nm as the reference wavelength. To allow for the fact that the absolute level of fluorescence at 430 nm is so small, we placed a N.D. 1 (10% transmission) filter in front of the 500 nm exciting light. On average we found, using nigericin calibrations in the intact cell (see below for methods), that changing the pH from 5·5 to 8·5 had the following effects on fluorescence: 430 nm, 22 ± 2% decrease ($n = 8$); 450 nm, $12{\cdot}3 \pm 0{\cdot}7$% increase ($n = 3$); 500 nm, 580 ± 66% ($n = 8$) increase (all figures, mean ± S.E.M.).

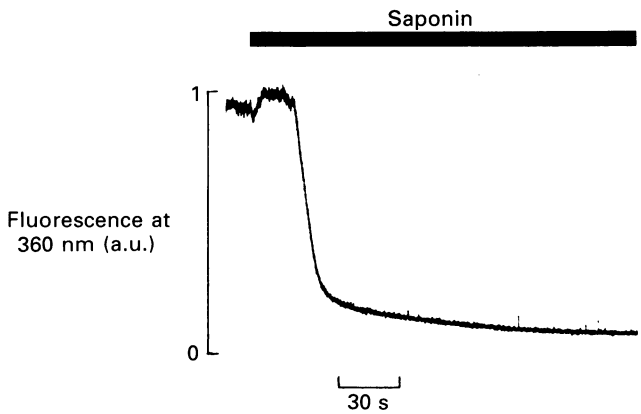

Fig. 3. The effects of saponin on the fluorescence of Fura-2 in the cell. The trace shows fluorescence excited at 360 nm. Saponin (50 μg ml^{-1}) was added for the period shown above.

Calibration of BCECF. Calibration of BCECF was done by equalizing pH_i and pH_o using the K^+–H^+ exchanger nigericin. At the end of each experiment the cell was superfused with a solution containing (in mM): KCl, 140; $MgSO_4$, 1·2; KH_2PO_4, 1·2; HEPES, 10; and 10 μM-nigericin. This was made up to each of the following values of pH: 5·5, 7, 8·5. The effects of this on the excitation spectrum measured in a single cell are shown in Fig. 4*A*. This was obtained on a fluorescence microscope fitted with an excitation monochromator (courtesy of Dr R. Jacob). The trace-labelled control shows the spectrum before adding nigericin. The other traces show the fluorescence in the presence of nigericin at various pH values. Inspection of this record shows that pH_i before adding nigericin was about 7·1. In this experiment the whole spectrum was measured. In most experiments, using the spinning wheel, fluorescence (F) was measured by excitation at 430, 450 and 500 nm. The effects of nigericin are shown in Fig. 4*B*. The pH can be calculated from a ratio R (F_{500}/F_{430}) using the equation:

$$pH_i = pK + \log \frac{R - R_{min}}{R_{max} - R} + \log (F_{430.min}/F_{430.max}),$$

where R_{min} is the ratio at extreme acid pH and R_{max} that at extreme alkaline pH. The pK was calculated from the nigericin calibration and was found to be $6{\cdot}97 \pm 0{\cdot}03$. This equation is essentially the same as that given by Grynkiewicz *et al.* (1985) for the calibration of Fura-2 and is different from that given by Paradiso *et al.* (1987) who used an isosbestic wavelength as a reference thus obviating the need for the third term on the right-hand side of the above equation. The nigericin method implicitly assumes that, in the presence of this compound, intracellular and extracellular pH are equal. We have therefore compared this method of calibration with one which involves the addition of mixtures of weak acids and bases (Eisner, O'Neill & Valdeolmillos, 1987). In general, good agreement was obtained between these methods.

Preparation of single cells. In most of the experiments the cells were made according to the

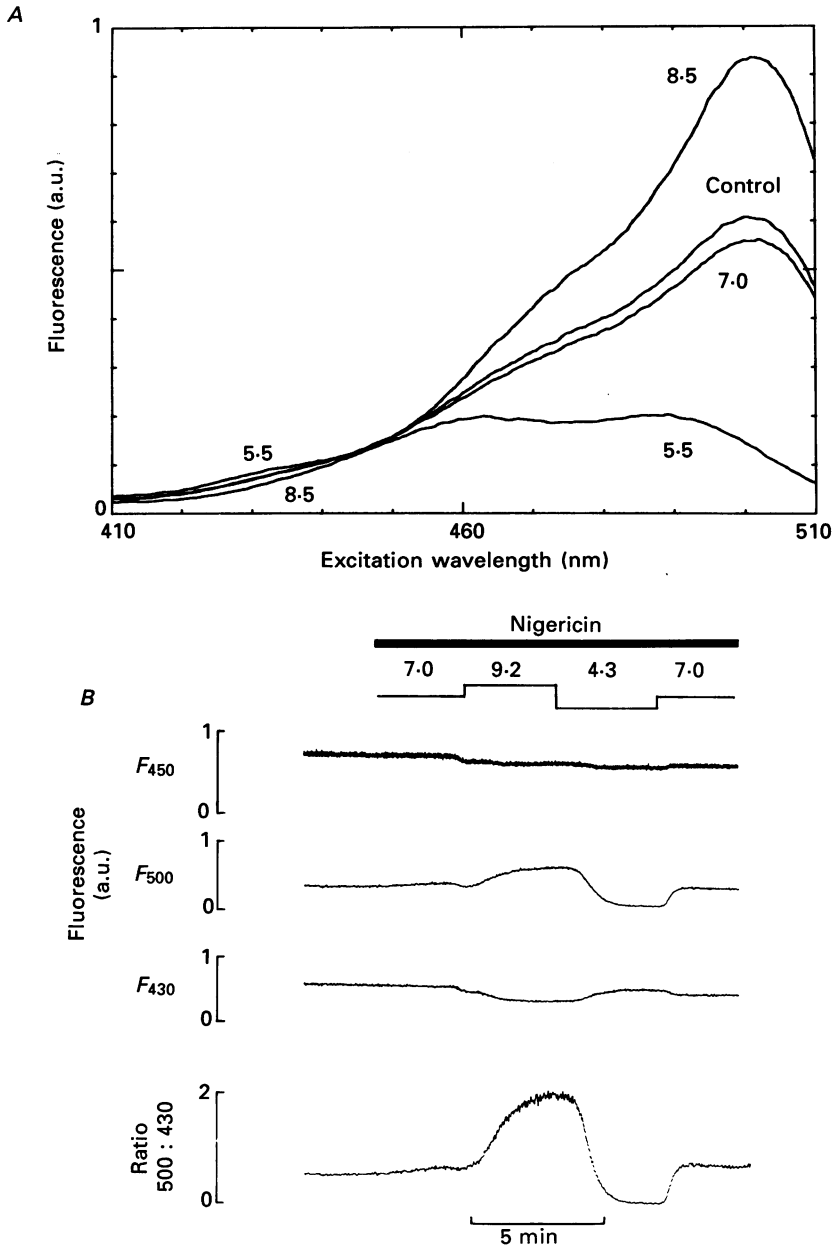

Fig. 4. Intracellular calibration of BCECF. *A*, spectra determined in a single cell. Ordinate, fluorescence intensity in arbitrary units (a.u.); abscissa, excitation wavelength. The trace labelled 'control' was obtained in the absence of nigericin. The other traces show the effects of nigericin- (10 μM) containing solutions with pH set equal to 8·5, 7·0 and 5·5. *B*, typical experimental calibration. This was obtained using excitation at (from top to bottom) 450, 500 and 430 nm using the spinning wheel. The bottom trace shows the ratio of fluorescence at 500 to that at 430 nm. The cell was initially in a control solution. Nigericin was added as shown and the pH of the calibrating solution changed between 7·0, 9·2 and 4·3. All calibrating solutions contained 140 mM-K^+. Note that, as in the other experiments in this paper, the 500 nm filter has a N.D. 1 filter in front of it thus decreasing the intensity of the signal to 10% (see Methods).

method of Powell, Terrar & Twist (1980) as modified and given to us by Dr C. H. Fry (St Thomas's Hospital, London). Briefly, after cervical dislocation, the heart was removed from the animal and perfused through the aorta with the nominally calcium-free HEPES-buffered solution described below. After perfusion for 1–2 min to clear the heart of blood, collagenase (Cooper Biomedical Type II) was added to a final concentration of 0·2 mg ml^{-1}. Calcium was added to this solution to increase the concentration by 25 μM. Perfusion was carried on for about 30 min. Then the heart was cut down and chopped into small pieces using fine scissors. The pieces of tissue were gently triturated through a plastic pipette and cells released by this process were, after gentle centrifugation, resuspended and stored in the standard solution containing 250 μM-calcium. In more recent experiments a different protocol given to us by Dr J. R. Berlin (Department of Physiology, University of Maryland School of Medicine) was used. The above calcium-free solution is perfused through the heart at a rate of about 10 ml min^{-1}. After 50 ml has been perfused 0·1 mg ml^{-1} of protease (Sigma Type XIV), 1 mg ml^{-1} collagenase (Cooper Biomedical Type II) and calcium to a final concentration of 100 μM are added. The heart is perfused with this solution for 4–5 min. Cells are released, resuspended and stored as described above.

Measurement of cell length. Cell length was measured routinely by placing a linear photodiode (LSC/30D: United Detector Technology, 12525 Chadron Avenue, Hawthorne, CA 90250, USA) on the video monitor. Contraction of the cell changed the distribution of light falling on the photodiode. The video signal was also routinely recorded to check the accuracy of this signal. Frame-by-frame measurements of cell length showed that the diode signal gave a good measure of the time course of the twitch. This method suffered sometimes from artifactual baseline changes. Therefore in Figs 13 and 14 we have shown cell length measured from the video records.

Solutions. All solutions were made with Milli-Q grade water. The control solution contained (in mM): NaCl, 133·5; KCl, 4; NaH_2PO_4, 1·2; $MgSO_4$, 1·2; HEPES, 10; glucose, 11·1; $CaCl_2$, 2; titrated to pH 7·4 with NaOH. All experiments were performed at 30 °C.

Measurement of lactic acid production. Cell suspensions were incubated in 1 ml Eppendorff test tubes for periods from 5 to 15 min in the presence or absence of cyanide. After centrifuging, the lactic acid content in the supernatant was assayed by measuring the absorbance of NADH produced from NAD (nicotinamide–adenine dinucleotide) in the presence of lactic dehydrogenase.

RESULTS

The effects of pH on contraction

Previous work has suggested that cyanide decreases contraction amplitude by decreasing intracellular pH (e.g. Allen *et al.* 1985). As a prelude to studying the effects of CN^- on single cells, we have therefore investigated the effects of intracellular pH on contraction. This was done in the experiment of Fig. 5*A* by adding the weak base NH_4Cl at constant external pH. This produces an alkalosis (due to rapid entry of NH_3) before pH_i slowly recovers (due to entry of NH_4^+). The subsequent removal of NH_4Cl produces an intracellular acidosis (as NH_3 leaves) followed by a slow recovery of pH_i. It is possible to calculate the intracellular buffering power from the initial change of pH_i produced by NH_4Cl. In this figure a value of 39·6 mmol (pH unit)$^{-1}$ is obtained. An average value of $25·6 \pm 3·8$ was obtained in seven cells. This value is less than that obtained in multicellular preparations and compares with that of 18 mM obtained previously in isolated guinea-pig myocytes (Bountra, Powell & Vaughan-Jones, 1987). This figure also shows that the changes of pH_i are accompanied by changes of contraction: an alkalosis increases contraction; an acidosis decreases contraction. This correlation between pH_i and contraction is in agreement with previous work on multicellular preparations (Vaughan-Jones, Eisner & Lederer, 1987). Figure 5*B* shows the quantitative relationship between contraction and pH_i. It is clear that there is a hysteresis

between contraction and pH_i. A similar hysteresis is seen in the Purkinje fibre (Vaughan-Jones *et al.* 1987). In that work there existed the possibility that the hysteresis was produced by diffusion delays between the surface cells (from which pH_i was measured) and the bulk of the preparation (which contributes to tension production). This possibility can be rejected for the present work on single cells.

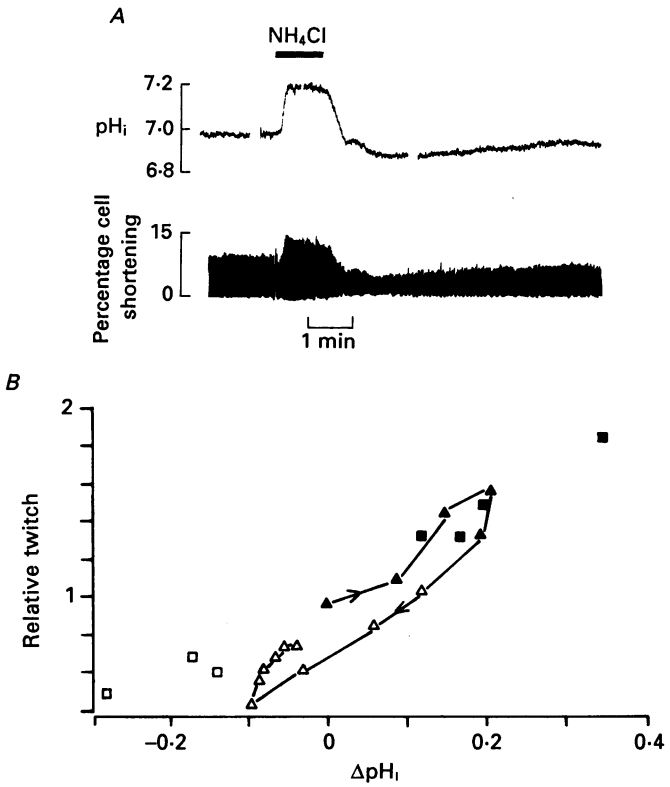

Fig. 5. The relationship between pH_i and contraction. *A*, original record. Traces show: top, pH_i; bottom, contraction. NH_4Cl (5 mM) was added for the period indicated. The two gaps in the record denote periods when the filters were changed in order to measure the reference wavelength. *B*, quantitative dependence of contraction on pH_i. The ordinate plots contraction relative to that in the control solution. The abscissa is the change in pH_i from the control. The triangles were obtained from the experiment of *A*. The filled triangles were obtained in the presence of NH_4Cl (note that the point at 0 ΔpH_i and twitch = 1·0 was obtained before adding NH_4Cl). The squares show points corresponding to the maximum acidosis and alkalosis produced in other experiments.

Figure 5*B* also shows points obtained from other experiments. These show only the maximum alkalosis and acidosis produced by a given exposure to NH_4Cl and the corresponding effects on contraction.

The effects of cyanide alone

The effects of CN^- (2 mM) on contraction were variable. Contraction increased in seventeen of thirty-one cells and remained the same in three. A decrease of contraction was seen in eleven. The experiment illustrated in Fig. 6*A* shows one of

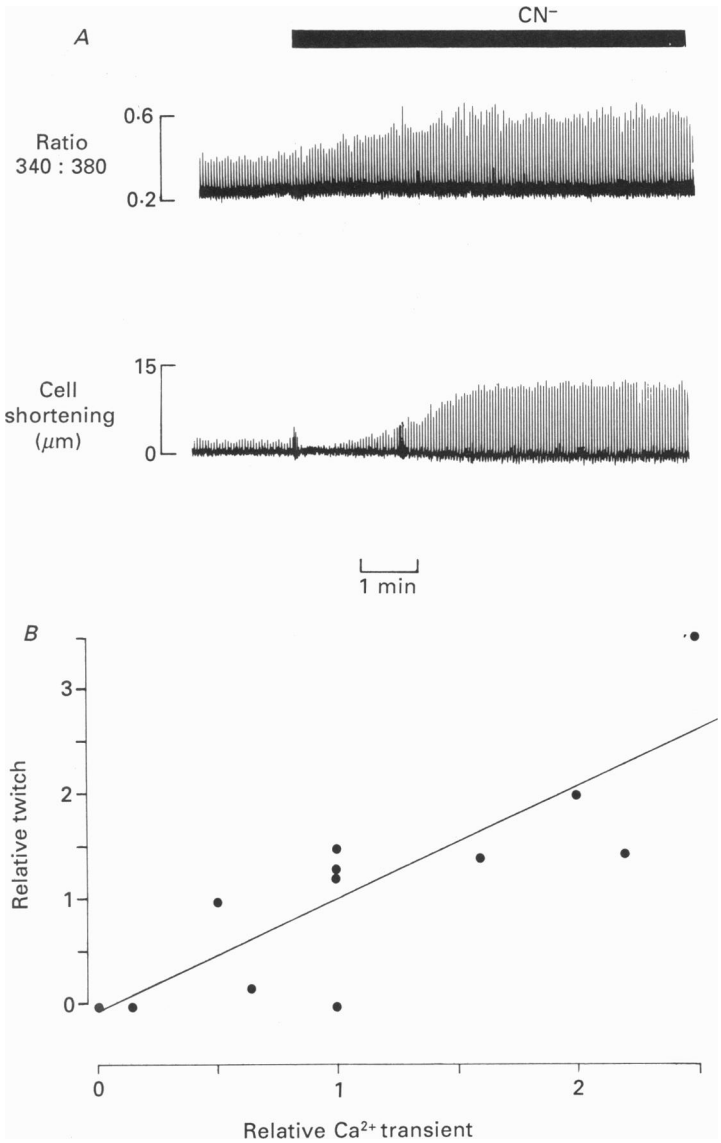

Fig. 6. The effects of CN^- on $[Ca^{2+}]_i$ and contraction. *A*, specimen original experiment. Traces show: top, the 340:380 nm ratio; bottom, contraction (0·1–10 Hz). CN^- (2 mM) was added for the period shown above. *B*, pooled data. The effects of CN^- on contraction (ordinate) and the magnitude of the systolic Ca^{2+} transient (abscissa) are shown. The equation of the regression line shown is $y = 1{\cdot}024x - 0{\cdot}073$ ($r^2 = 0{\cdot}66$), the intercept of this line with the y-axis is not significantly different from the origin ($P > 0{\cdot}8$).

the larger increases of contraction produced by CN^-. The apparent initial transient decrease in contraction is an artifact associated with a change in solution level. The Fura-2 record shows that the increase of contraction is accompanied by a small increase in the magnitude of the Ca^{2+} transient. Figure 6*B* shows data from all the experiments in which $[Ca^{2+}]_i$ was measured. It is clear that CN^- can either increase

or decrease the magnitude of the Ca^{2+} transient. This is accompanied by corresponding changes of contraction. It is particularly noteworthy that the regression line predicts that for no change in the systolic Ca^{2+} transient there will be no change in contraction. This is very different from the case in much previous work where CN^- decreases contraction at constant $[Ca^{2+}]_i$ (Allen & Orchard, 1983). Despite this

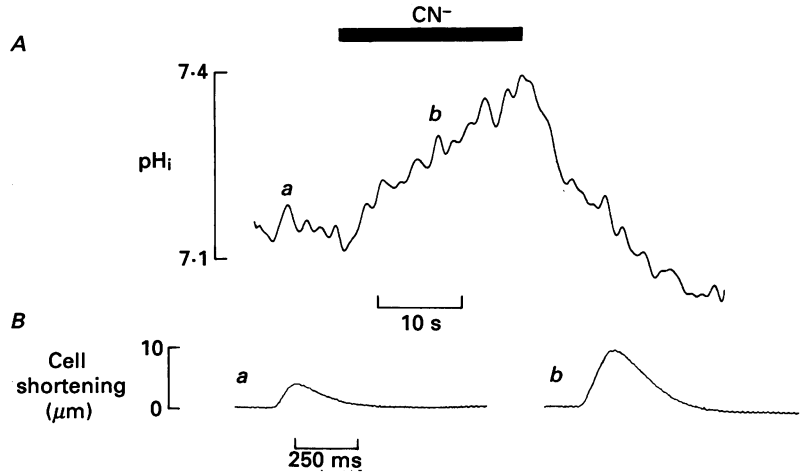

Fig. 7. The effects of CN^- on pH_i and contraction. *A*, time course of change of pH_i measured from the 500:430 nm ratio. *B*, specimen twitches obtained at the times indicated on *A*.

correlation between $[Ca^{2+}]_i$ and contraction, there are clearly some experiments in which large changes of contraction amplitude on exposure to CN^- are unaccompanied by changes of systolic $[Ca^{2+}]_i$. One explanation for the change of contraction without a change of $[Ca^{2+}]_i$ is suggested by the experiment illustrated in Fig. 7. In this experiment pH_i was measured using BCECF. The addition of CN^- produced an alkalosis of about 0·24 pH units which was accompanied by an increase of contraction. Comparison with Fig. 5 shows that this alkalosis alone would be expected to increase contraction by about 60%, a value reasonably close to that observed.

Much previous work has found that CN^- addition causes an intracellular acidosis that can be attributed to lactic acid production. In twenty-three cells exposed to CN^- an acidosis was seen in only five. We have checked to see whether isolated myocytes produce lactic acid in the presence of cyanide. Addition of cyanide to a suspension of cells increased lactic acid production from undetectably low levels (< 5 μmol (g dry weight)$^{-1}$ min^{-1}) to an average of 25 μmol g^{-1} min^{-1}. We have found similar values of lactate production in Langendorff-perfused rat hearts exposed to CN^-. These values are also in agreement with those found by others in single cells (Haworth, Hunter, Berkoff & Moss, 1983).

The effects of cyanide when glycolysis is inhibited

Effects on contraction

We have used three methods to inhibit glycolysis: (i) prolonged incubation in glucose-free solutions; (ii) the addition of iodoacetate to inhibit glyceraldehyde-

3-phosphate dehydrogenase; (iii) the addition of 2-deoxyglucose (DOG) which is converted to deoxyglucose-6-phosphate, an inhibitor of phosphoglucoisomerase (Noltman, 1972). No qualitative difference was found between these manoeuvres. In the experiment illustrated in Fig. 8*A*, the cell was first exposed to 0·5 mM-iodoacetate. The subsequent addition of cyanide produced the following pattern of

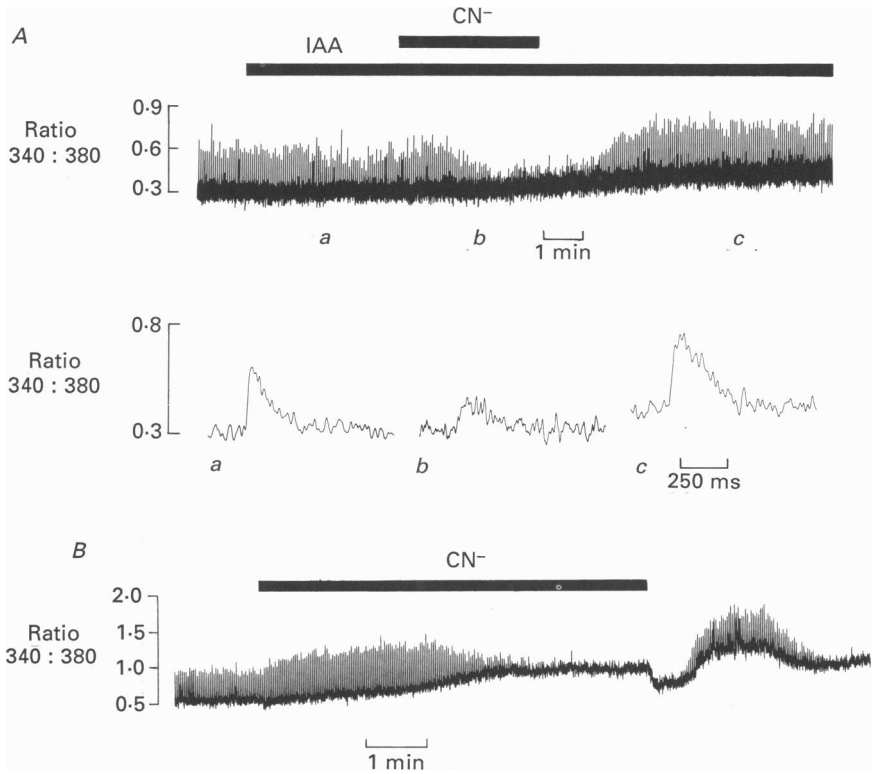

Fig. 8. The effects of cyanide on $[Ca^{2+}]_i$ with glycolysis also inhibited. *A*, the effects of brief exposure to CN^-. The top trace shows the time course of change of $[Ca^{2+}]_i$ as estimated from the 340:380 ratio. The bottom shows specimen-averaged ($n = 8$) transients from the periods shown above. Iodoacetate (IAA, 0·5 mM) and cyanide (CN^-, 2 mM) were applied for the periods shown. *B*, partial recovery from metabolic inhibition. The trace shows $[Ca^{2+}]_i$ as estimated from the 340:380 nm ratio. 2-Deoxyglucose (10 mM) had been present for 20 min before the record began. CN^- (2 mM) was added for the period shown.

events. Over 2 min there was a decrease in the magnitude of the Ca^{2+} transient which was accompanied by a small increase of the diastolic level of $[Ca^{2+}]_i$. This was seen in all similar experiments. When CN^- was removed the Ca^{2+} transients reappeared and were larger than control. This was associated with an increase of diastolic $[Ca^{2+}]_i$. The degree of reversibility of this metabolic blockade was variable and depended on the duration of exposure to CN^-. Figure 8*B* shows the effects of a longer exposure to CN^- after prolonged exposure to DOG. Diastolic $[Ca^{2+}]_i$ increased significantly during exposure to CN^-. On removal of CN^- $[Ca^{2+}]_i$ first decreased and then rose to higher levels before eventually recovering. Figure 8*B* also shows a small

increase in the magnitude of the systolic Ca^{2+} transient on adding CN^-. This was seen in thirteen out of forty-three experiments. In Fig. 9, the addition of CN^- in the presence of iodoacetate rapidly abolished the twitch and the Ca^{2+} transient. The specimen Ca^{2+}transients show no change in time course. This abolition of the Ca^{2+} transient was then followed by the development of a diastolic contracture. This

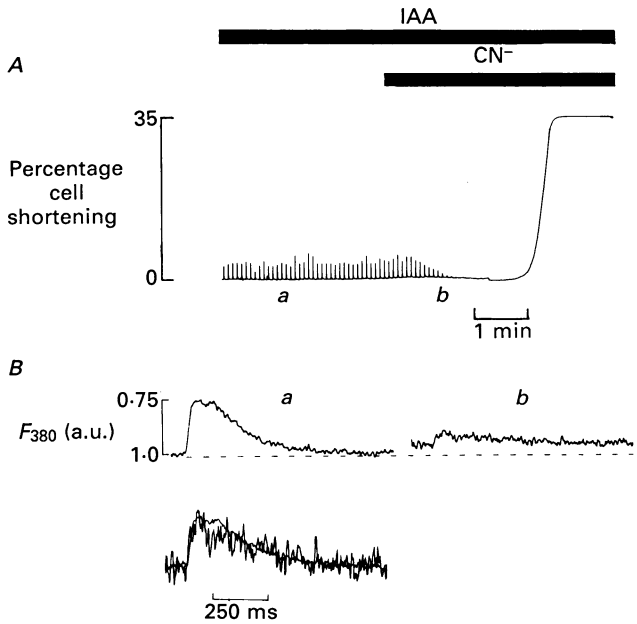

Fig. 9. Irreversible effects of metabolic inhibition. *A*, time course of changes of contraction. Iodoacetate (IAA, 0·5 mM) and cyanide (CN^-) were applied for the periods shown. *B*, specimen-averaged ($n = 8$ for transient, *a*, $n = 4$ for transient *b*) calcium transients from the periods shown in *A*. The transients are shown normalized below. Note that, in order to improve signal-to-noise the calcium transients were recorded only at 380 nm (see Methods). The fluorescence records have therefore been inverted so that an upward deflection still corresponds to an increase of $[Ca^{2+}]_i$.

diagram (and also Fig. 14) shows therefore that diastolic $[Ca^{2+}]_i$ rises before the cell develops a diastolic contracture. This is in disagreement with previous work where diastolic $[Ca^{2+}]_i$ increases *after* the onset of the contracture (Cobbold & Bourne, 1984; Smith & Allen, 1988).

The experiments presented above have used the acetoxymethyl (AM) ester of Fura-2. This potentially suffers from the problems discussed in the Methods section. We have therefore attempted to use the free acid of Fura-2 introduced into the cell via a patch pipette. In four successful experiments we were able to compare the signals with those obtained with *in vitro* calibration. We obtained a mean resting $[Ca^{2+}]_i$ of 93 ± 12 nm. Figure 10 shows the effect of metabolic inhibition on such a cell. It is again apparent that $[Ca^{2+}]_i$ begins to increase before the cell develops a contracture. A further increase of $[Ca^{2+}]_i$ is then associated with the development of the contracture. At the end of the period of metabolic inhibition $[Ca^{2+}]_i$ has reached a level of about 2 μM. The mean level of $[Ca^{2+}]_i$ after metabolic blockade was 1·7 μM.

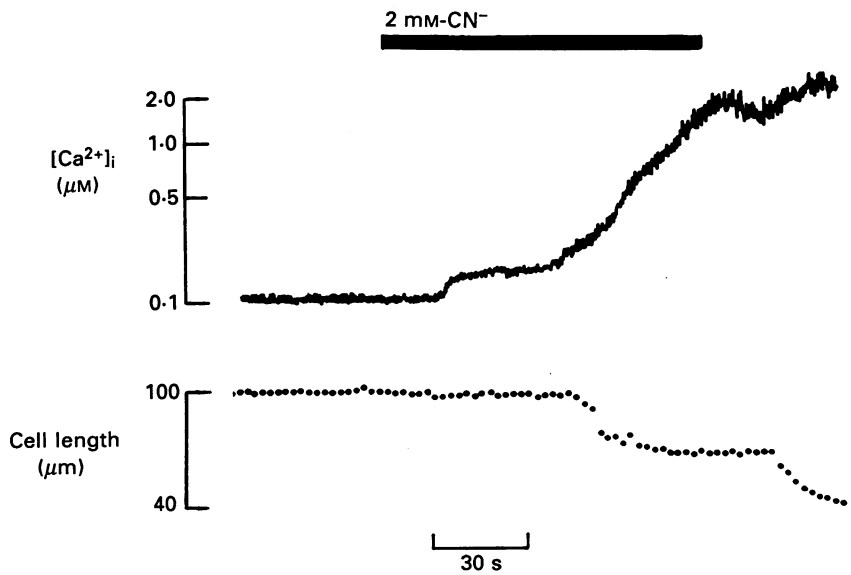

Fig. 10. The effects of metabolic inhibition on $[Ca^{2+}]_i$ studied using the free acid of Fura-2. Traces show: top, $[Ca^{2+}]_i$ calibrated from an *in vitro* calibration; bottom, contraction. The cell had been exposed to 5 mM-2-deoxyglucose (DOG) for 10 min before the record began. CN^- was applied for the period shown above.

Effects on pH_i

We have studied the effects of combined inhibition of aerobic and anaerobic metabolism on pH_i. Figure 11 shows that the addition of CN^- in the presence of iodoacetate produced a transient alkalosis followed by the development of an acidosis, accompanied by the abolition of contraction. The removal of CN^- restored both pH_i and contraction. Figure 12 shows the response of a cell to a longer exposure to CN^-. It is clear that a significant acidosis develops before the onset of diastolic contracture.

Contracture and pH_i

It is apparent (Figs 9 and 14) that diastolic $[Ca^{2+}]_i$ rises almost to control systolic levels before any diastolic shortening occurs. This suggests that the myofilament Ca^{2+} sensitivity is decreased at this time. Such an effect could be a consequence of the marked acidosis since acidosis is known to decrease the Ca^{2+} sensitivity in skinned cardiac cells (Fabiato & Fabiato, 1978).

The experiment illustrated in Fig. 13 was designed to test whether the intracellular acidosis could be responsible for the inhibition of contracture in the presence of elevated $[Ca^{2+}]_i$. CN^- was applied (in the absence of glucose) until a significant acidosis had developed. At this time, twitch amplitude had decreased but there was no change of diastolic length. NH_4Cl was then added to produce an intracellular alkalosis. This was accompanied by a diastolic contracture and a small, transient increase of the twitch. This result is in accord with the suggestion that the intracellular acidosis produced by CN^- inhibits diastolic contraction.

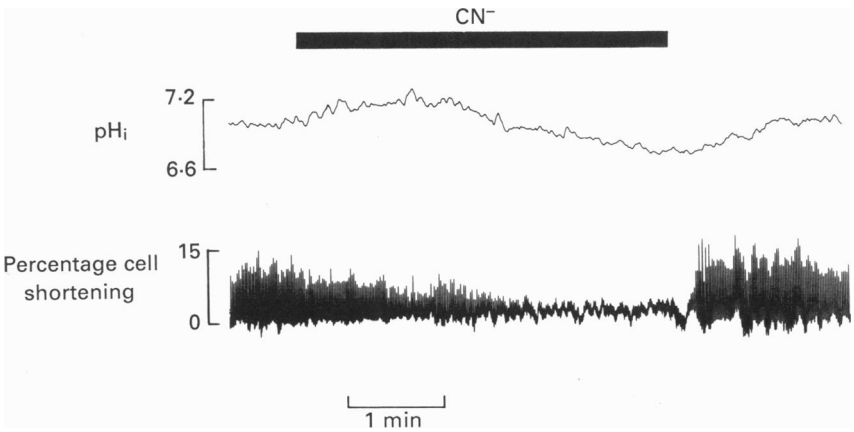

Fig. 11. The effects of metabolic inhibition on pH_i and contraction. Traces show: top, pH_i measured from the 500:430 ratio; bottom, contraction. The contraction record has been bandpass filtered (0·1–10 Hz) in order to eliminate slow baseline drifts. However the video recording showed that there was no change in cell diastolic length during this period. The cell had been exposed to iodoacetate (0·5 mM) for 10 min before the record began. Cyanide (CN^-, 2 mM) was applied for the period shown above.

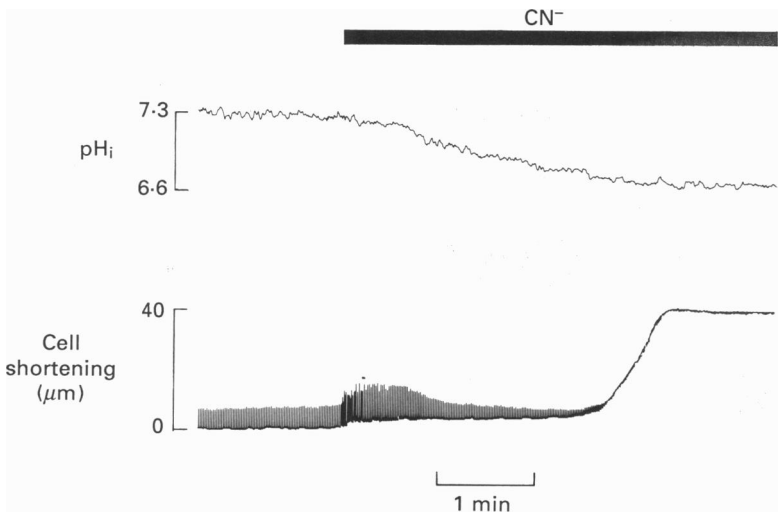

Fig. 12. The relationship between changes of pH_i and mechanical events. Traces show: top, pH_i calculated from the 500:430 ratio; bottom, cell length. The cell had been stored in a glucose-free solution for 40 min. Cyanide (CN^-) was applied for the period shown.

Contracture and $[Ca^{2+}]_i$

If increased $[Ca^{2+}]_i$ is responsible for contracture shortening, then lowering $[Ca^{2+}]_i$ should relax the cell. However, as shown in Fig. 14, decreasing $[Ca^{2+}]_i$ (by removing extracellular Ca^{2+}) did not relax contracture, even though $[Ca^{2+}]_i$ fell to a lower level than that present at the onset of contracture. This hysteresis between $[Ca^{2+}]_i$ and contracture implies the existence of a Ca^{2+}-independent mechanism.

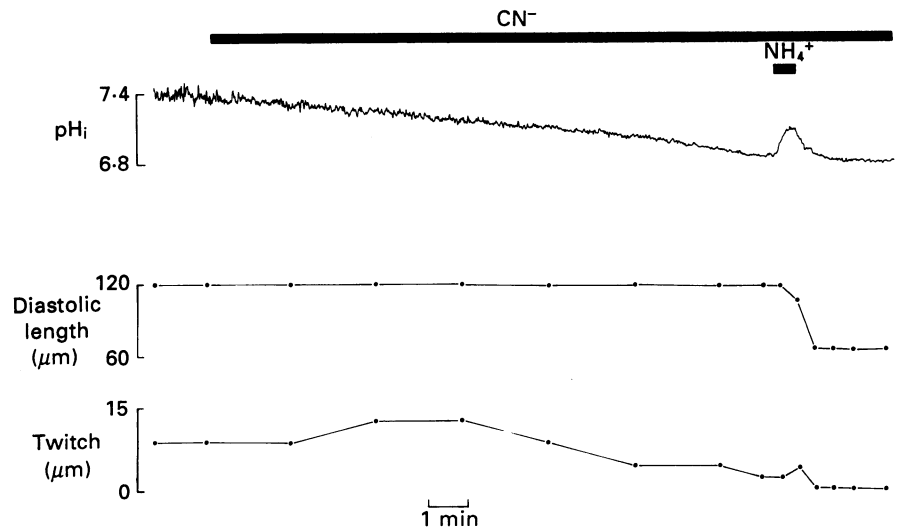

Fig. 13. The effects of NH_4Cl on contraction and pH_i in the presence of cyanide. Traces show: top, pH_i; middle, diastolic cell length; bottom, twitch shortening. The cell had been stored in a glucose-free solution for 35 min before the record began. Cyanide (CN^-, 2 mM) and NH_4Cl (NH_4^+, 10 mM)were applied for the periods shown.

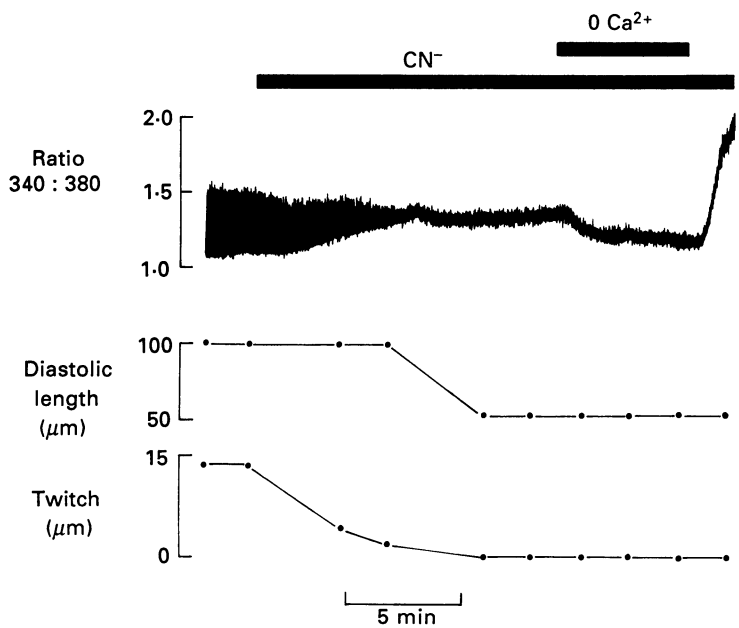

Fig. 14. The effects of removal of extracellular calcium on $[Ca^{2+}]_i$ and contraction. Traces show: top, 340:380 ratio; middle, diastolic cell length; bottom, twitch shortening. The cell had been exposed to DOG for 30 min before the record began. Cyanide (CN^-) and a calcium-free solution (0 Ca^{2+}) were applied for the periods shown.

Factors influencing the acidosis

One consistent observation was that the acidosis produced by metabolic inhibition developed faster in stimulated than quiescent cells. In the experiment of Fig. 15 the cell was initially stimulated at 1 Hz. The addition of cyanide in the absence of glucose resulted in a gradual acidosis. However when the stimulation rate was increased to

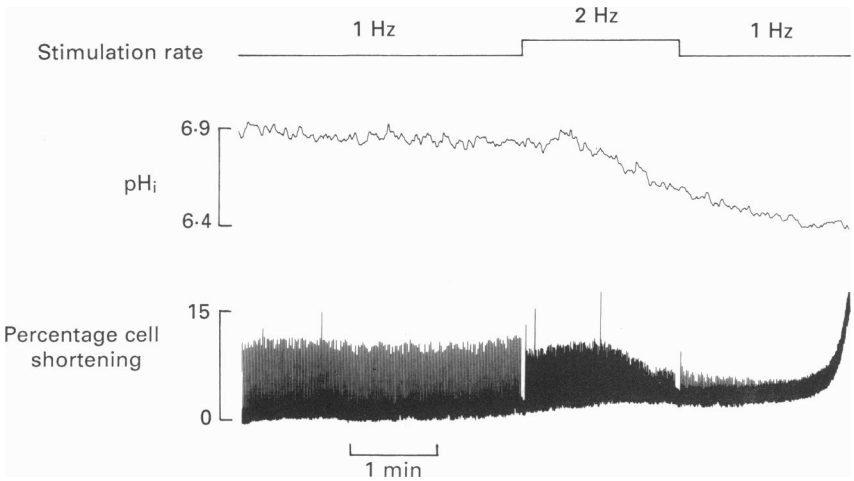

Fig. 15. The effects of stimulation rate on the development of the intracellular acidosis produced by metabolic inhibition. Traces show: top, pH_i; bottom, cell length. The rate of stimulation is shown above. The cell had been stored in a glucose-free solution for 50 min and exposed to cyanide (2 mM) for 5 min before the record began.

2 Hz the rate of acidification increased dramatically. Increasing the rate of stimulation initially decreased the twitch. This is also seen in control cells and reflects the negative force–frequency relation seen in rat heart cells (Capogrossi, Kort, Spurgeon & Lakatta, 1986). However contraction then declined precipitously before the cell went into contracture.

In all the experiments seen so far there has been a rough correlation between the decrease of pH_i and the increase of resting $[Ca^{2+}]_i$. Increase of $[Ca^{2+}]_i$ can decrease pH_i in cardiac muscle (e.g. Vaughan-Jones *et al.* 1983; Ellis & MacLeod, 1985). It is possible that the intracellular acidosis produced by metabolic blockade might result directly from an increase of $[Ca^{2+}]_i$. In order to test this possibility we have examined the effects on pH_i of removing external Ca^{2+} during metabolic blockade. In the experiment illustrated in Fig. 16*A* metabolic blockade produced an acidosis. Subsequent removal of external Ca^{2+} did not, however, restore pH_i. Similarly, if the whole experiment was performed in a Ca^{2+}-free solution, metabolic blockade still produced a diastolic contracture and acidosis (not shown). Another possible explanation for the intracellular acidosis was investigated in the experiment illustrated in Fig. 16*B*. It has been shown that the membrane permeability to potassium ions increases during metabolic inhibition (Vleugels, Vereeke & Carmeliet, 1980; Taniguchi, Noma & Irisawa, 1983). It is therefore also possible that it becomes

more permeable to H^+ ions and that this contributes to the acidosis. We have investigated this by changing external pH to 8·4. Under these conditions, so long as pH_i is less than 7·0, the passive driving force on protons will be outward as long as the membrane potential is no more negative than −84 mV. Therefore an increase of permeability to protons should result in an intracellular alkalosis. Figure 15*B* shows, however, that this extracellular alkalosis does not reverse the intracellular acidosis.

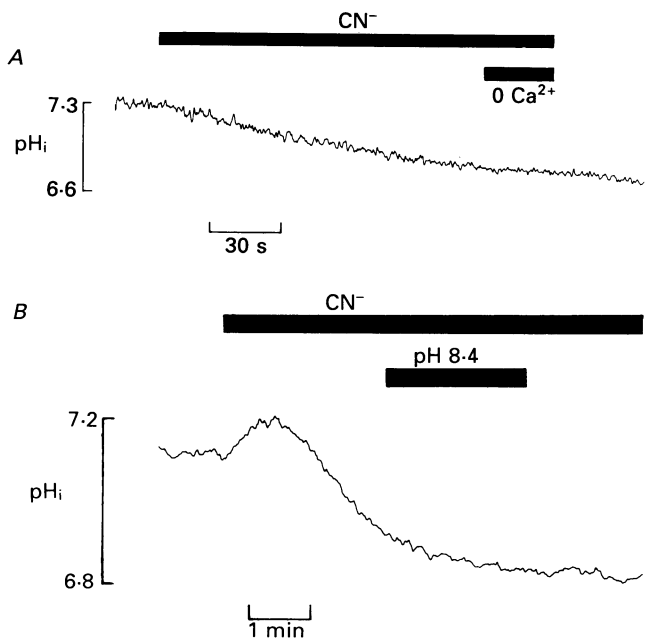

Fig. 16. The lack of effect of changing external calcium and pH on the acidosis produced by metabolic inhibition. *A*, the effects of removal of external calcium. The trace shows pH_i calculated from the 500:430 ratio. The cell had been exposed to DOG (10 mM) for 25 min before the record began. External calcium was removed (1 mM-EGTA) as shown. *B*, the effects of changing external pH. The cell had been exposed to DOG (10 mM) for 30 min before the record began. The solution (control pH 7·4) was changed to one buffered to 8·4 for the period shown above.

The effects of iodoacetate alone

The above experiments have shown that cyanide has different effects when applied in the presence of iodoacetate than when applied alone. Iodoacetate alone produces qualitatively the same effects as cyanide plus iodoacetate although the effects develop more slowly (Fig. 17). There is, however, considerable variability in the time course of the effects. Figure 17*A* shows the effects of adding iodoacetate alone on $[Ca^{2+}]_i$ and contraction. This produces a gradual *increase* in contraction with little change in the magnitude of the Ca^{2+} transient. The twitch then declines along with an increase of resting $[Ca^{2+}]_i$ before a contracture occurs. Figure 17*B* shows that iodoacetate alone produces an intracellular acidosis. If cells were exposed to deoxyglucose alone then the same qualitative effects as produced by iodoacetate were

seen although they developed more slowly. An explanation of these effects may be that prolonged blockade of glycolysis by the inhibition of the enzyme glucose-6-phosphate transmutase will cause a build-up of sugar phosphates within the cell and effectively trap all the cellular phosphate (Pirolo & Allen, 1986) which will eventually cause cell death. Such phosphate-trapping may also increase the Ca^{2+} sensitivity of

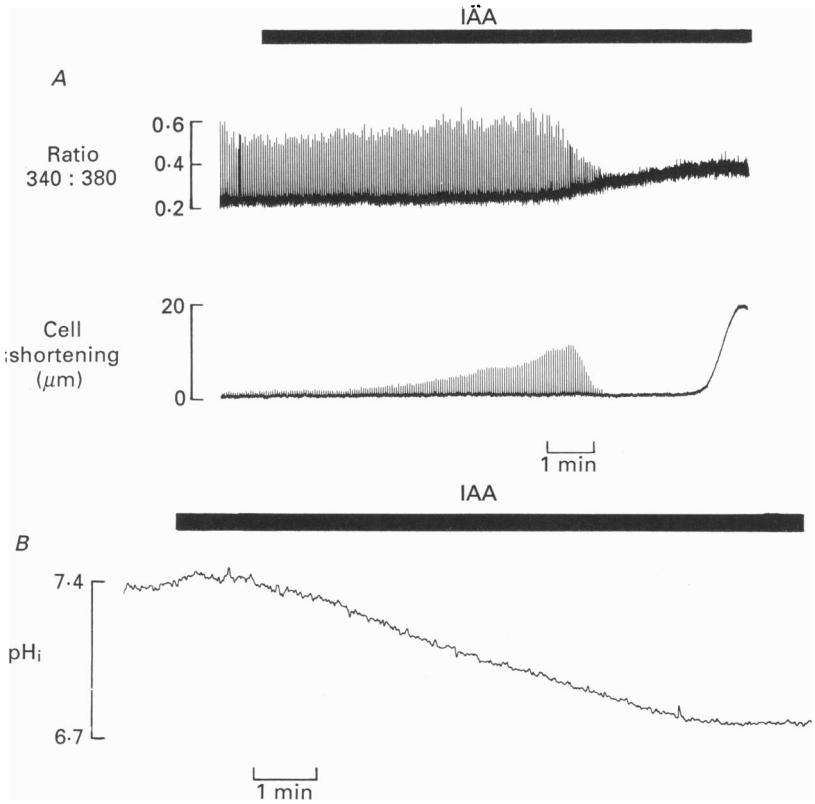

Fig. 17. The effects of iodoacetate alone. *A*, effects on $[Ca^{2+}]_i$ and contraction. Traces show: top, $[Ca^{2+}]_i$; bottom, contraction. Iodoacetate (IAA, 0·5 mM) was applied for the period shown above. *B*, effects on pH_i. Iodoacetate (IAA, 0·5 mM) was applied for the period shown.

the contractile apparatus (Kentish, 1986) and thus account for much of the increase of contraction. Finally simple exposure to a glucose-free solution did not produce these effects.

DISCUSSION

The effects of cyanide alone

Effects on pH_i

Previous work on multicellular preparations has shown that cyanide application produces an intracellular acidosis attributed to formation of lactic acid by anaerobic glycolysis. This is sometimes preceded by an intracellular alkalosis attributed to

break-down of phosphocreatine (Allen *et al.* 1985; Fry, Harding & Mounsey, 1987; Ellis & Noireaud, 1987). In contrast, in the present study, CN^- did not usually produce an intracellular acidosis. There are at least three explanations for this result. (i) The myocytes may be doing less work than the multicellular preparations and therefore have a lower glycolytic rate. While this may explain the difference with respect to work on beating Langendorff hearts, it does not account for the observation that an acidosis still occurs in quiescent preparations (Ellis & Noireaud, 1987). Furthermore, stimulation of lactic acid production by CN^- appears to be as great in our single cells as in the intact heart. (ii) It may be that a lactate-independent mechanism contributes to the acidosis produced by CN^- alone since we have shown that metabolic inhibition can acidify the cell under conditions when lactic acid production is inhibited. (iii) Intracellular accumulation of lactic acid in multicellular preparations may be a significant cause of CN^--induced acidosis whereas, in single cells, easier diffusion away from the cells may limit the magnitude of this accumulation. There are two restraints to lactic acid leaving the preparation: (i) diffusion across the cell membrane; (ii) diffusion through the extracellular space of the tissue to the bulk solution. The latter barrier may be significant in multicellular preparations. We assume that a multicellular preparation such as a papillary muscle can be represented as a cylinder of radius a, producing lactic acid at a rate r per unit weight. Then if the diffusion constant for lactate is D it can be shown (Hill, 1928) that the mean extracellular lactate concentration will be $r\,a^2/8D$. A free solution diffusion constant of 5×10^{-6} cm^2 s^{-1} was estimated from Kushmerick & Podolsky (1969) and reduced to 1×10^{-6} cm^2 s^{-1} to allow for tortuosity of the extracellular space (Safford & Bassingthwaighte, 1977). Taking a value of lactic acid production of 3 μmol g^{-1} min^{-1} (Pirolo & Allen, 1986) gives a mean extracellular lactate concentration of about 16 mM for a 1 mm diameter fibre and of about 4 mM for a fibre of 0·5 mm diameter. Therefore in a multicellular preparation, the intracellular concentration of lactate will be higher than it would be if the cells were isolated. In fact the effect will be even greater since the extracellular lactic acid will acidify the extracellular space which will further inhibit lactic acid efflux (Deuticke, 1982) thus increasing intracellular lactic acid concentration.

Measurements of pH in multicellular preparations are generally made from surface cells which would be expected to have lower extracellular lactic acid concentrations than those calculated above. It should, however, be noted that some commonly used cardiac preparations and, in particular, the Purkinje fibre are surrounded by a barrier to diffusion. This barrier has been calculated to be such that (for a fibre of the size used in voltage-clamp experiments) a 1 mM concentration difference of an ion is required to allow a total current of 1·6 nA (Attwell, Eisner & Cohen, 1979). These calculations were made for K^+ currents but should also be applicable to lactate. We have no figures for the total lactate production by a Purkinje fibre but, under anaerobic conditions, it should be sufficient to provide ATP for the Na^+–K^+ pump. Under steady-state conditions the Na^+–K^+ pump produces a current of about 10 nA in a Purkinje fibre (Eisner, Lederer & Vaughan-Jones, 1981). Since the production of one molecule of lactate produces one ATP there must be an efflux of lactate from the preparation equivalent to 10 nA. This will therefore require a concentration gradient across the diffusion barrier of about 6 mM. This calculation is approximate and presumably underestimates the lactic acid production but does suggest that even surface cells may be exposed to high lactate concentrations.

Effects on contraction and intracellular Ca^{2+}

The present results show that the application of cyanide has variable effects on contraction. This can be largely attributed to changes in the systolic Ca^{2+} transient. In multicellular preparations a transient increase of contraction is sometimes produced by CN^- alone (Allen & Orchard, 1983; Allen *et al.* 1985) where it is not accompanied by a change in the Ca^{2+} transient and has been attributed to an intracellular alkalosis. CN^- has also been shown to increase the size of the twitch in frog atrial muscle (Chapman, 1973). The alkalosis in the present work may therefore also be responsible for some of the increase of contraction. However recently, Allen, Lee & Smith (1988) have shown that, under some conditions, CN^- can increase the Ca^{2+} transient in ferret papillary muscles despite a decrease of contractility. They suggested that this increase of the Ca^{2+} transient resulted from an intracellular acidosis. In some cells we have also seen an increase in the Ca^{2+} transient which is associated with an increase of contraction. The major difference between the present results and the previous work is the fact that CN^- does not decrease contraction to the same level as is seen in multicellular preparations. The mean contraction in CN^- was 127 ± 27 % (mean $\pm$ S.E.M.; $n = 31$) of the control. The decrease of force seen in multicellular preparations appears to result from the combination of an increase of inorganic phosphate (P_i) and a decrease of pH_i decreasing the force produced for a given $[Ca^{2+}]_i$ (Allen *et al.* 1985). It is therefore possible that the fact that CN^- alone does not produce an acidosis could account for the lack of fall of contraction in single cells. However Eisner *et al.* (1987*a*) showed, in ferret hearts, that most of the CN^--induced decrease of contraction was due to the increase of P_i rather than the fall of pH_i. It may be that CN^- increases P_i less in single cells, perhaps because the unloaded cell shortening consumes less ATP. Alternatively, Cooke & Pate (1985) have recently found that while P_i depresses isometric force in skeletal muscle fibres, the maximum velocity of shortening is unaffected. It is therefore possible that a rise in $[P_i]_i$ will not affect the extent of shortening in these isolated cardiac cells.

The effects of cyanide with glycolysis inhibited

Effects on pH

When glycolysis was inhibited, the addition of CN^- produced a large, rapidly developing acidosis. This acidosis was of similar magnitude irrespective of whether glycolysis was inhibited by exposure to glucose-free solutions, application of 2-deoxyglucose, or application of iodoacetate. Since these will result respectively in the accumulation of inorganic phosphate, 2-deoxyglucose-6-phosphate, and glyceraldehyde-3-phosphate, it is unlikely that the acidosis results from the accumulation of any particular metabolite. The acidosis seen here contrasts with previous work. Allen *et al.* (1985) found that inhibition of glycolysis *abolished* the acidosis produced by CN^- thus supporting the hypothesis that the CN^--induced acidosis resulted from lactic acid accumulation. In contrast, the results of Ellis & Noireaud (1987; their Fig. 9) show that the CN^--induced acidosis is essentially unaffected by glycolytic inhibition. Our experiments also show that the acidosis develops more quickly as stimulation rate is increased. This does not, however, appear to be a consequence of changes of $[Ca^{2+}]_i$.

There are at least two explanations for the acidosis produced by metabolic inhibition. (i) It has recently been suggested (Wu & Vaughan-Jones, 1988) that Na^+–H^+ exchange is inhibited by metabolic inhibition in cardiac Purkinje fibres. Although we cannot exclude this possibility, we have noted (not shown) that the acidosis produced by metabolic inhibition develops much more quickly than that produced by inhibiting Na^+–H^+ exchange with amiloride analogues. (ii) The acidosis may result from protons released by break-down of ATP. It has been calculated that, at neutral pH, the hydrolysis of 1 mol of ATP (to ADP and P_i) will give about 0·8 mol of protons (e.g. Wilkie, 1979). In cardiac muscle, however, ADP does not accumulate during metabolic inhibition and undergoes further hydrolysis. The complete hydrolysis of ATP to phosphate and adenine can be shown to release about one proton at pH 7·0 (Wolfe, Gilbert, Brindle & Radda, 1988). Set against this is the fact that hydrolysis of phosphocreatine (PCr; to creatine and P_i) absorbs about 0·35 protons per PCr. If we assume that complete metabolic inhibition results in the hydrolysis of all the ATP and PCr the overall proton release will depend on the initial concentrations of ATP and PCr. A [PCr]:[ATP] ratio of 2 has been found in glucose-perfused rat hearts (Bailey, Radda, Seymour & Williams, 1982) corresponding to initial concentrations of about 14 mM-PCr and 7 mM-ATP. These were completely hydrolysed during ischaemia. From the figures given above this would be expected to release about 2 mmol of protons. Given a buffering power of about 25 mmol/pH unit (this paper; Bountra, Powell & Vaughan-Jones, 1987; Wolfe *et al.* 1988) this would produce an acidosis of 0·1 pH units. The acidosis may be even greater since the decrease of [ATP] may decrease the buffering power. The conclusion then is that break-down of ATP can account for a fraction of the intracellular acidosis. Of course, if the control [PCr] is less in isolated cells than in the intact heart, the acidosis attributable to PCr and ATP hydrolysis will be increased.

The above calculation may also explain why some previous work (Allen *et al.* 1985) has not found an intracellular acidosis during metabolic inhibition. In their study perfused ferret hearts were made hypoxic while glycolysis was inhibited either by previous glycogen depletion or by the addition of deoxyglucose. Under these conditions [PCr] fell from 19 to 2 mM and [ATP] from 5·4 to 3·3 mM. This can be calculated to *absorb* a net amount of 3·8 mM-protons thus accounting for the observed lack of an intracellular acidosis. The general conclusion is that, since the net proton release depends on the difference between two larger quantities (PCr hydrolysis consuming protons *versus* ATP hydrolysis liberating them), then small changes in either will have large effects on the net pH change. In all studies metabolic inhibition results in the disappearance of most PCr. What varies between studies is the amount of ATP consumed and therefore the amount of the acidosis.

Effects on contraction and $[Ca^{2+}]_i$

The present results show that the addition of cyanide with glycolysis inhibited eventually decreases the magnitude of the systolic calcium transients and increases diastolic $[Ca^{2+}]_i$. These results are in agreement with those obtained with aequorin (Allen & Orchard, 1983; Smith & Allen, 1988). It is likely that the reduction of systolic $[Ca^{2+}]_i$ results from the abolition of the action potential (Lederer, Nichols & Smith, 1987). The present results show that the increase of diastolic $[Ca^{2+}]_i$ precedes

the development of the contracture. This is in disagreement with previous work using aequorin (Allen & Orchard, 1983; Cobbold & Bourne, 1984; Allshire, Piper, Cuthbertson & Cobbold, 1987; Smith & Allen, 1988) which found that the contracture preceded any rise of $[Ca^{2+}]_i$ suggesting that the contracture resulted from a fall of ATP (producing rigor) rather than from an increase of $[Ca^{2+}]_i$. It should, however, be noted that aequorin is not very sensitive at normal resting $[Ca^{2+}]_i$ and that a rise in diastolic $[Ca^{2+}]_i$ may have been missed. The experiments using the Fura-2 free acid show that the present results are not simply a consequence of problems of Fura-2 in intracellular organelles. Qualitatively, one of the most striking differences between our results and those of Allshire *et al.* (1987) is that, in the previous work, $[Ca^{2+}]_i$ rose abruptly *after* the cell had gone into contracture. This was interpreted as resulting from the low ATP contracture consuming what ATP remains and therefore interfering with Ca^{2+} regulation. The steady rise in $[Ca^{2+}]_i$ in our experiments is not consistent with such a positive feed-back mechanism. That $[Ca^{2+}]_i$ can rise to appreciable levels before a contracture develops can be explained by the accompanying intracellular acidosis and its depressant effect on myofilament Ca^{2+} sensitivity. This is supported by the observation that, during metabolic blockade, intracellular alkalinization produces an immediate contracture (Fig. 13). The present results do show, however, that a Ca^{2+}-independent component is involved in causing contracture (Fig. 14) since, once the contracture has developed, it is not reversed by lowering $[Ca^{2+}]_i$.

It has been suggested (Smith & Allen, 1988) that the increase of diastolic $[Ca^{2+}]_i$ in metabolic blockade is due to a decreased efflux of Ca^{2+} on Na^+–Ca^{2+} exchange as a consequence of elevated $[Na^+]_i$. The increase of $[Na^+]_i$ is thought to result from inhibition of the Na^+–K^+ pump by a fall of $[ATP]_i$ as evidenced by the onset of rigor contracture. It is therefore worth noting that, in the present study, $[Ca^{2+}]_i$ increases before rigor.

Conclusions

We have shown that inhibition of oxidative phosphorylation does not decrease contraction. This difference, and the lack of an acidosis, contrasts with previous work on multicellular preparations and may be related to the geometry of the single cells, or to differences in metabolic demand.

After inhibition of anaerobic metabolism, the application of CN^- produces a contracture which is preceded by a rise of diastolic $[Ca^{2+}]_i$. During this period at least three factors are responsible for regulating the contracture. (i) $[Ca^{2+}]_i$ increases, possibly due to inhibition of Ca^{2+} pumping out of the cell. (ii) Metabolic inhibition directly decreases [ATP]. These first two factors will promote a contracture. (iii) An intracellular acidosis will decrease the contracture.

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