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Cytosolic free Ca^{2+} in single rat heart cells during anoxia and reoxygenation

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Free Ca^{2+} in the cytosol ($[Ca^{2+}]_i$) of individual rat ventricle cells injected with aequorin was measured under anoxia. In glucose-free medium myocytes spontaneously shortened after about 60 min, although $[Ca^{2+}]_i$ was still at or near resting levels. However, within minutes a net inward movement of Ca^{2+} across the sarcolemma developed and $[Ca^{2+}]_i$ began to rise. Provided oxygen was readmitted before $[Ca^{2+}]_i$ exceeded 2–3 μM, cells were able to restore $[Ca^{2+}]_i$ to resting levels through caffeine-sensitive sequestration of Ca^{2+} in the sarcoplasmic reticulum. We suggest that Ca^{2+}-independent shortening of anoxic cardiomyocytes reflects onset of rigor which triggers loss of $[Ca^{2+}]_i$ homoeostasis.

INTRODUCTION

On interruption of its oxygen supply, the heart must rely on glycolysis to sustain an intracellular phosphorylation potential consistent with survival (Williamson, 1966). However, substrate-level phosphorylation does not prevent a rapid decrease in phosphocreatine and a slower decrease in ATP during myocardial ischaemia (Hearse *et al.*, 1977) or anoxic perfusion (Kammermeier *et al.*, 1982). Eventually a point will be reached where the free energy of ATP hydrolysis is no longer sufficient to allow the ATPase-linked pumps to maintain essential ion gradients across cell membranes.

The possible role of $[Ca^{2+}]_i$ in damage to heart tissue caused by hypoxia and reoxygenation has received much attention (see Poole-Wilson *et al.*, 1984, and references therein). Nevertheless the information yielded by multicell models, whether intact tissue or suspensions of dissociated cells, is limited by (i) the rapidity of changes caused by reoxygenation, (ii) cell–cell heterogeneity and asynchrony, and (iii) uncertainty as to how total Ca^{2+} content is related to $[Ca^{2+}]_i$. It has now become possible to micro-inject single mammalian cardiomyocytes with aequorin and obtain a continuous non-perturbing measure of $[Ca^{2+}]_i$ (Cobbold & Bourne, 1984). In experiments described below, we obtained parallel measurements of $[Ca^{2+}]_i$ and cell morphology in individual rat heart myocytes during anoxia and reoxygenation. Some of the data have been presented elsewhere in abstract from (Allshire *et al.*, 1986).

EXPERIMENTAL

Cells

Hearts of adult male Wistar rats (body wt. 250–350 g) were dissociated by a recirculating Langendorff perfusion with collagenase (type II, Worthington), essentially as described by Piper *et al.* (1982). The preparation contained a high proportion of cells which remained rod-shaped and quiescent in a medium with 1.8 mM-Ca^{2+}. Cells were maintained at 37 °C under 5% CO_2 in air in medium 199 with Earle's salts (Flow Laboratories) with 26.2 mM-$NaHCO_3$, and were used within 8 h of isolation.

Micro-injection

Samples from the stock cell suspension were diluted 10-fold in medium 199 buffered with 20 mM-$NaHCO_3$ and 30 mM-Hepes/NaOH (pH 7.4) and supplemented with 0.1% (w/v) fatty acid-free bovine serum albumin and 1% type IX agarose (Sigma). Individual cells were held inside micro-slides (0.1 mm path length; Camlab, Cambridge, U.K.) in a similar medium containing gelled type VII agarose (Sigma), and micro-injected to less than 1% of cell volume with dialysed aequorin (40–50 mg/ml) in 150 mM-KCl/1 mM-Pipes/NaOH (pH 7.3)/0.1 mM-EDTA/25 μM-EGTA, essentially as described by Cobbold *et al.* (1983). Micro-pipettes were filled with aequorin immediately before each injection simply by dipping the tip into a droplet of aequorin stock held under liquid paraffin. Before the beginning of the experiment, injected cells were returned to the incubator for at least 30 min to allow them to recover fully. Most remained quiescent and elongated, with distinct sarcomeres.

Measurement of $[Ca^{2+}]_i$ and cell shape

The micro-slide containing the injected cell was transferred to a 0.3 ml polished stainless-steel cup with glass windows top and bottom and superfused at 370 μl/min and 37 °C with a medium containing 125 mM-NaCl, 2.6 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 1.0 mM-$CaCl_2$ and 10 mM-Hepes/NaOH (pH 7.4), bubbled with 'O-free' N_2 (O_2 < 5 p.p.m.; B.O.C.). To remove O_2, 200 ml portions of medium supplemented with 1.5 μM-resazurin (Sigma) as redox indicator and 0.83 mM-cysteine were autoclaved, then kept under O_2-free N_2 at positive pressure. Since at 37 °C and pH 7.3 the colourless dihydroresorufin derivative of resazurin begins to oxidize to resorufin (pink) at an O_2 partial pressure of about 21.9 μPa (2.16×10^{-10} atm), this set an upper limit on the O_2 content of the medium.

Abbreviations used: $[Ca^{2+}]_i$, concentration of free Ca^{2+} in the cytosol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Anoxic medium was supplied to the cup through steel tubing with articulated joints of relatively gas-impermeable butyl XX rubber. Reoxygenation or additions to the medium were carried out in the supply vessel. Glucose was omitted from the superfusate throughout.

Once the cell was in place, the cup was positioned directly below (about 8 mm clearance) a low-noise photomultiplier (bialkali photocathode, 10 mm diameter, type 9789A; E.M.I.) kept at 4 °C (see Cobbold *et al.*, 1983), and photon counts were relayed to a Sirius micro-computer for storage and analysis. A beam of red light from a light-emitting diode (output 35 μW at 820 nm, product 309-515 from Radiospare Components) was directed downwards through the cup via an angled plastic optical fibre positioned just below the shutter. The resulting image of the cell was magnified with a 60 × microscope objective (Nikon) beneath the lower window of the cup, and transmitted via a red-sensitive TV camera (TM-36K; Pulnix, Sunnyvale, CA, U.S.A.) to an external monitor and video recorder. To record cell morphology, no more than a 5 s break in measuring aequorin light was generally needed. An air-actuated shutter protected the photomultiplier during viewing of the cell. Cell outlines were traced from 35 mm photographs of the monitor screen.

At the end of each experiment any remaining aequorin was discharged by lysing the cell in distilled water.

Aequorin calibration

The rate of aequorin consumption as a function of free Ca^{2+} concentration was calibrated *in vitro* over the pCa range 5–8 in 150 mM-KCl/10 mM-EGTA/10 mM-Pipes/NaOH (pH 7.4)/5 mM free Mg^{2+} as described by Cobbold *et al.* (1983). The specific relation obtained was then used as the basis for converting raw count rates from aequorin-containing cells into values for $[Ca^{2+}]_i$. Unfortunately, direct measurements of cytosolic free Mg^{2+} in rat heart are not available. However, calibration at a single free Mg^{2+} concentration appears to be justified by measurements in sheep ventricle with micro-electrodes (Hess *et al.*, 1982). These workers recorded a value of 3.1 mM free Mg^{2+}, which did not rise significantly even when the main intracellular Mg^{2+} ligand, ATP, was depleted by using CN^-, iodoacetate or 2,4-dinitrophenol, suggesting that the lower affinity of ATP-hydolysis products for Mg^{2+} is largely offset by their greater combined activity.

Statistical analysis

Mean resting $[Ca^{2+}]_i$ values were derived from maximum-likelihood estimators of the mean of the count distribution (sampled at 1 s intervals), truncated if necessary to remove high counts caused by phasic contractions. Comparisons of means between control and test periods, or between test periods and a given $[Ca^{2+}]_i$, were computed from count histograms by likelihood ratio tests with the chi-square approximation. The post-shortening test periods quoted as having $[Ca^{2+}]_i$ significantly below a given value were such that, in all instances of probe periods sufficiently long to overcome Poisson noise and containing at least one point of the test period, $[Ca^{2+}]_i$ was significantly low. The last such probe period extended beyond the quoted time point towards the subsequent $[Ca^{2+}]_i$ rise, so that the best estimate of the time at which $[Ca^{2+}]_i$ actually rose above the given value would be somewhat later. Goodness-of-fit tests confirmed the fit of the histograms of counts to Poisson (or truncated Poisson) distributions.

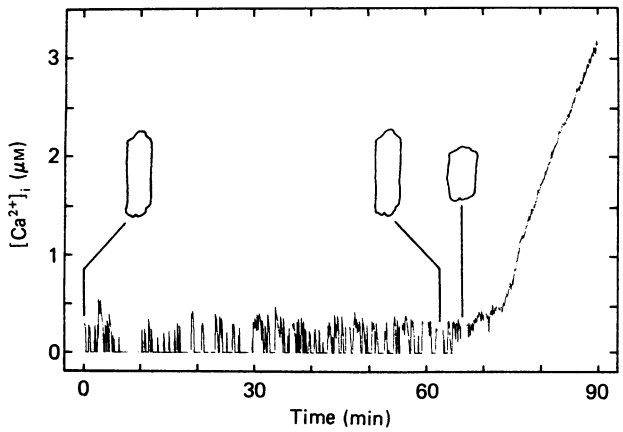

Fig. 1. $[Ca^{2+}]_i$ in a rat cardiomyocyte superfused with anoxic medium containing 1 mM-Ca^{2+} but lacking glucose

Outlines depict cell shapes recorded at the points indicated. Conditions and calibration of the aequorin signal are described in the Experimental section. Total aequorin light in the cell was 1.2×10^5 counts. Data are exponentially smoothed, and plotted with time constants of 20 s for aequorin consumption rates corresponding to $[Ca^{2+}]_i$ < 560 nM, and 5 s for consumption rates corresponding to $[Ca^{2+}]_i$ > 560 nM. The mean $[Ca^{2+}]_i$ values over the 1000 s before shortening and over 60 s after shortening, 183 and 258 nM respectively, were not significantly different ($P > 0.05$). $[Ca^{2+}]_i$ was significantly below 350 nM for 78 s and below 400 nM for 109 s after shortening was first detected ($P < 0.05$).

RESULTS

$[Ca^{2+}]_i$ was measured in a series of 22 unstimulated cardiomyocytes superfused with an anoxic medium containing 1 mM-Ca^{2+} but lacking glucose. Fig. 1 shows the record from a cell qualitatively representative of the group. After prolonged anoxia (35–130 min, median 64 min) the elongated cells spontaneously shortened to about 60% of their original length (median value; range 33–70%) over 10–30 s. Distinct sarcomeres were no longer visible and, whereas ripples of reversible contraction (phasics) had occasionally propagated along the fully elongated cells, shortened cells were completely quiescent as long as anoxia was maintained.

Throughout the period before shortening occurred, $[Ca^{2+}]_i$ remained low: mean values from 15 cells ranged between 145 and 259 nM, of which the overall mean was 195 ± 8 nM (S.E.M.). But after shortening $[Ca^{2+}]_i$ began to rise, exceeding 1 μM 3–5 min later. Up to several minutes elapsed between detection of shortening and onset of the $[Ca^{2+}]_i$ rise, but the true lag was probably somewhat longer, because cell shape was monitored only at 3–5 min intervals and the shortening event itself was often missed. In three cells where the $[Ca^{2+}]_i$ rise was delayed and which contained relatively large amounts of aequorin (1×10^5–2×10^5 photon counts), detailed analysis of the signal showed that $[Ca^{2+}]_i$ before and after shortening had not changed significantly ($P > 0.05$), and was still less than 450 nM ($P < 0.05$) 1 min after shortening was detected. In separate experiments a cell

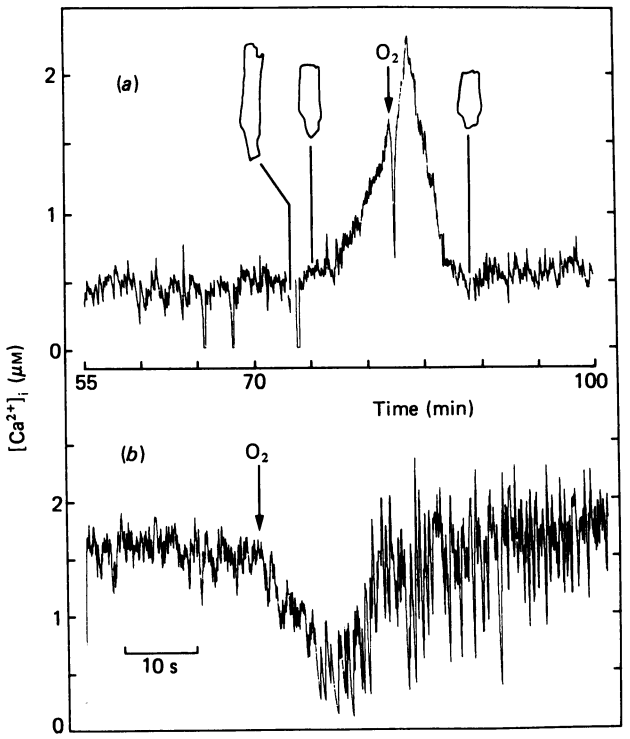

Fig. 2. Reoxygenation after $[Ca^{2+}]_i$ had begun to rise above resting values in a shortened cardiomyocyte

In (*a*), O_2 reached the cup at the point indicated, but we have no measure of the rate at which dissolved O_2 appeared in the immediate environment of the cell. Total aequorin counts were 6.3×10^4. Time constants: 20 s for $[Ca^{2+}]_i < 700$ nM, 3 s for $[Ca^{2+}]_i > 700$ nM. (*b*) Detail of the reoxygenation event, (time constant 0.2 s).

superfused with 0.2 mM-Ca^{2+} resembled the control cells superfused with 1 mM-Ca^{2+} in the time at which it shortened and the brief delay before $[Ca^{2+}]_i$ rose. Three cells superfused with lower concentrations of Ca^{2+} shortened at 85 and 90 min with 0.1 mM-Ca^{2+}, and at 88 min with no added Ca^{2+}, but no effect on resting $[Ca^{2+}]_i$ was seen. The $[Ca^{2+}]_i$ rise was both delayed and slowed: 4 min after shortening was first detected, $[Ca^{2+}]_i$ remained significantly below 300 nM ($P < 0.05$). It appears therefore that the $[Ca^{2+}]_i$ rise after shortening reflects a net movement of Ca^{2+} into the cell from the external space.

Re-introduction of O_2 after various periods of anoxia provoked a spectrum of responses. Two cells not yet shortened after 50 and 60 min respectively remained fully elongated and quiescent with a resting $[Ca^{2+}]_i$ upon reoxygenation. By contrast, reoxygenation of shortened cells invariably triggered a 3–5 min episode of spontaneous mechanical activity (3–4 Hz) paralleled by oscillations of $[Ca^{2+}]_i$. Overall, the consequences for cell shape and Ca^{2+} homoeostasis depended on $[Ca^{2+}]_i$ at the time of reoxygenation. Fig. 2 shows the behaviour of one of a group of four truncated cells reoxygenated while $[Ca^{2+}]_i$ was still less than 1.5 μM. No further change in cell shape occurred and, after the period of twitching and $[Ca^{2+}]_i$ oscillations, $[Ca^{2+}]_i$ returned to resting values for the remainder of the experiment (at least 30 min). This ability of both shortened and unshortened cells to maintain $[Ca^{2+}]_i$ at resting values for long periods after O_2 was re-admitted indicates that shortening did not result from exhaustion of endogenous substrate to support phosphorylation of ADP, although shortening may coincide with a thermodynamic limit on glycolysis that is relieved on resumption of aerobic metabolism.

The outcome of reoxygenation while $[Ca^{2+}]_i$ was in the range 1.5–3 μM was more variable: of the four cells in this category examined, one remained rod-shaped, but the other three promptly rounded up. Mechanical activity was in the form of twitching or isotropic 'squirming' respectively, after which $[Ca^{2+}]_i$ was restored to resting values. However, 5–20 min later $[Ca^{2+}]_i$ began to rise again and the remaining rod-shaped cell rounded up. Eventually all cells developed large blebs (> 3 μm) of the sarcolemma.

A third category comprised 14 cells reoxygenated only after $[Ca^{2+}]_i$ already exceeded 5 μM. All rounded up immediately when exposed to O_2, and, although in some cases the $[Ca^{2+}]_i$ rise was temporarily slowed or even partly reversed, it soon re-established itself, and the cells went on to bleb and lyse.

Taken together these experiments show that further shape changes by truncated cells required both O_2 and micromolar $[Ca^{2+}]_i$, suggesting that a Ca^{2+}-sensitive mechanism was involved that depended on a high free energy of ATP hydrolysis. Two particular points may be pertinent to the response of intact myocardium to anoxia

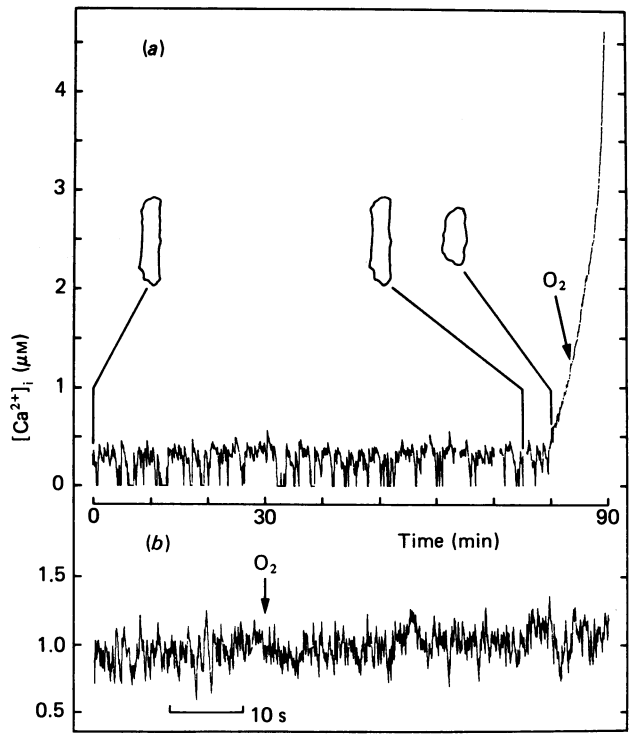

Fig. 3. Effect of caffeine on $[Ca^{2+}]_i$ during anoxia and reoxygenation

The cell was superfused throughout with 5 mM-caffeine and 1 mM-Ca^{2+}; O_2 reached the cup at the point indicated. Total aequorin counts were 1.0×10^5. In (*a*) time constants: 20 s for $[Ca^{2+}]_i < 590$ nM, 5 s for $[Ca^{2+}]_i > 590$ nM. (*b*) Detail of the reoxygenation period (time constant 0.2 s).

and reoxygenation. First, the shortening event marks a dramatic change in cardiomyocytes' response to reoxygenation, from quiescence to a period of rapid spontaneous twitching accompanied by $[Ca^{2+}]_i$ oscillations that may be related to the arrhythmic twitching which occurs in reoxygenated heart. Second, the ability of cells to restore Ca^{2+} homoeostasis upon reoxygenation is exceeded once $[Ca^{2+}]_i$ reaches about 5 μM; sustained recovery in isolated cardiomyocytes was only possible when $[Ca^{2+}]_i$ was less than 1.5 μM.

To establish what role the Ca^{2+}-sequestering activity of the sarcoplasmic reticulum played in recovery of resting $[Ca^{2+}]_i$ on reoxygenation, five cells were superfused throughout the experiment with 5 mM-caffeine as well as 1 mM-Ca^{2+}. Under these conditions, reoxygenation no longer provoked either mechanical activity of shortened cells or reversal of the $[Ca^{2+}]_i$ rise, even when $[Ca^{2+}]_i$ at the point of reoxygenation was no more than about 1 μM (Fig. 3). Rather, the $[Ca^{2+}]_i$ rise continued and cells eventually rounded and lysed. Evidently, a sarcoplasmic reticulum competent to remove Ca^{2+} from the cytosol is essential to the recovery of Ca^{2+} homoeostasis. Consequently, the 3–5 μM limit on to recovery of Ca^{2+} homoeostasis under control conditions may reflect the maximum capacity of the reticulum in these cells to accumulate cytosolic Ca^{2+}, or perhaps a kinetic limit on Ca^{2+} accumulation owing to rate-limiting rephosphorylation of ADP exacerbated by the depletion of the total adenine nucleotide pool as a result of anoxia.

DISCUSSION

The essential finding of this study is that spontaneous shortening of single heart cells after prolonged anoxia (Haworth *et al.*, 1981; Stern *et al.*, 1985) is not due to any significant rise in $[Ca^{2+}]_i$ above resting values, but rather precedes it. A similar sequence has been seen in myocytes exposed to metabolic inhibitors (Cobbold & Bourne, 1984; Cobbold *et al.*, 1985).

What triggers cell shortening, then, if not Ca^{2+}? Interaction of actin and myosin to generate force is sensitive not only to Ca^{2+} through troponin C but also to ATP. In mixtures of actin and myosin *in vitro*, unless MgATP is sufficient to saturate the myosin fully, a net formation of actomyosin rigor complexes takes place independent of Ca^{2+}. Once they reach a critical concentration, these complexes 'switch on' the actin filaments to interact with remaining myosin–ATP, leading to contraction and hydrolysis of bound ATP (Bremel & Weber, 1972). This response is evident in digitonin-permeabilized cardiomyocytes, which contract independently of Ca^{2+} when ATP is decreased to 1–10 μM (Altschuld *et al.*, 1985). In both ischaemic heart (Hearse *et al.*, 1977) and anoxic cardiomyocytes (Piper *et al.*, 1984), large decreases in ATP have been reported, but whether the formation of rigor complexes in intact cells is determined by ATP free energy of hydrolysis or by P_i concentration is not yet clear (Kammermeier *et al.*, 1982; Kentish & Allen, 1986). Contracture in anoxic myocardium has been proposed to involve rigor, since heat output is so low as to preclude all but an abnormally slow turnover of cross-bridges (Holubarsch *et al.*, 1982) and hypoxic heart is unable to recover tension after a quick alteration in length (Ventura-Clapier & Vassort, 1981). In the present study the shortening of anoxic cardiomyocytes was independent of $[Ca^{2+}]_i$, and we suggest that it is due to the onset of rigor. A corollary of this inference, incidentally, is that if changes in $[Ca^{2+}]_i$ can be discounted, in myocytes at least, cell shape provides an index of cytosolic ATP.

The strict temporal relationship that we observed between shortening and the ensuing rise in $[Ca^{2+}]_i$ can be explained if the former is attributed to rigor. Rigor-induced contraction involves an activation of the myosin ATPase, which may deplete remaining ATP still further. This could decrease the free energy of ATP hydrolysis from the 45–50 kJ/mol at which contractile failure occurs in heart to less than the 40–45 kJ/mol required to drive the sarcolemmal and sarcoplasmic-reticulum Ca^{2+} pumps, and Na^+–Ca^{2+} exchange indirectly via the $Na^+ + K^+$-dependent ATPase (both estimates from Kammermeier *et al.*, 1982), thereby impairing Ca^{2+} homoeostasis. By restoring a high free energy of ATP hydrolysis through timely reoxygenation and resumption of oxidative phosphorylation of ADP, pumping of Ca^{2+} should resume and rigor complexes should dissociate, though cells not under any tension do not revert to their original elongated shape.

The key role played by the sarcoplasmic reticulum in restoring Ca^{2+} homoeostasis after reoxygenation is demonstrated by the ability of caffeine to prevent a reverse of the $[Ca^{2+}]_i$ rise. Despite the substantial capacity of isolated heart mitochondria for Ca^{2+} in the presence of P_i as permeant anion (see Saris & Åkerman, 1980), the caffeine experiment also rules out a significant role for this pool in effecting recovery of Ca^{2+} homoeostasis upon reoxygenation, at least in the early stages of the rise.

In summary, cardiac myocytes injected with aequorin spontaneously shorten before $[Ca^{2+}]_i$ rises to values required to activate the myofibrils. The results provide further support for an involvement of rigor in anoxic contracture of heart, and show that the shortening event triggers a departure from Ca^{2+} homoeostasis. Recovery of homoeostasis is possible if O_2 is reintroduced soon after shortening, whereupon the sarcoplasmic reticulum removes Ca^{2+} from the cytosol. The ability of caffeine to abolish this recovery showed that other Ca^{2+} pumps, mitochondrial and sarcolemmal, contribute relatively little to the process.

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