Evidence for rapid consumption of millimolar concentrations of cytoplasmic ATP during rigor-contraction of metabolically compromised single cardiomyocytes

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Cytoplasmic ATP can be measured continuously in single cardiac myocytes by monitoring the luminescence from microinjected firefly luciferase. We show here that the signals are markedly influenced by changes in cytoplasmic pH, and the calibration of the signals as ATP concentration is markedly affected by cytoplasmic protein. Measurements with a pH-sensitive fluorescent dye show that intracellular pH (pH_i) can be clamped at pH 7.08 by perfusing cells with a modified bicarbonate-buffered Krebs saline containing 92 mM NaHCO_3 and equilibrated with 20% CO_2 . Calibration of the firefly luciferase signal *in vitro* in the presence of high concentrations of BSA (180 mg/ml), to simulate the cytoplasmic protein concentration, revealed a shift in K_m (ATP) to 2 mM, from approx. 400 μM in the absence of

albumin in an identical ionic milieu. Luciferase measurements in pH-clamped cells show that metabolically poisoned isolated rat ventricle cardiomyocytes enter rigor at a cytoplasmic ATP concentration of between 1 and 2 mM. As the cells shorten in rigor, a process that is complete in 30–40 s, the cytoplasmic ATP concentration falls simultaneously to a level of typically 20 μM . When cyanide is removed 10 min later, to simulate reoxygenation, the signal recovers over a period of 2–3 min to a level approx. 70% of the original in the healthy cell. These studies indicate that rigor-mediated depletion of cytoplasmic ATP in metabolically poisoned cardiomyocytes is considerably more extreme than hitherto indicated.

INTRODUCTION

Single isolated ventricular cardiomyocytes from rat provide a model for the study of cellular and biochemical events after metabolic inhibition that circumvents problems of cell-to-cell heterogeneity and asynchrony, which are inherent in multicellular studies. Measurements of the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in single cardiomyocytes proved to be valuable in refuting the concept that metabolic inhibition promptly induced a loss of control of calcium homeostasis. Indeed, we now know that elevation of $[\text{Ca}^{2+}]_i$ in cardiomyocytes subjected to metabolic poisons or anoxia follows $[\text{Ca}^{2+}]_i$ -independent, rigor-mediated cell shortening [1–3]. To explain the relation between the sudden shortening of metabolically poisoned cardiomyocytes and the loss of ionic homeostasis the following working hypothesis was formulated [4]. $[\text{Ca}^{2+}]_i$ -Independent myocyte shortening (contraction) is caused by activation of myosin ATPase by rigor complexes, resulting in sudden depletion of cytoplasmic ATP to levels unable to sustain normal Na^+ and Ca^{2+} pumping. To test this hypothesis it was necessary to prove that myocyte shortening occurred simultaneously with a precipitous fall in cytoplasmic ATP concentration ($[\text{ATP}]_i$). A method based upon microinjecting isolated cardiomyocytes with the ATP-sensitive bioluminescent protein firefly luciferase was therefore developed [5]. Firefly luciferase, in the presence of ATP, Mg^{2+} , O_2 and luciferin, produces light. Thus by microinjecting firefly luciferase into single cardiomyocytes, and allowing luciferin to diffuse into the cell from an oxygen-containing medium, it is possible to monitor changes in $[\text{ATP}]_i$ during metabolic inhibition. In this way a sudden fall in $[\text{ATP}]_i$ has been shown to occur simultaneously with rigor-mediated cell shortening [5,6]. In similarly poisoned

hepatocytes the sudden fall in luciferase signal is not seen [7]. A deficiency with these results is that the luciferase luminescent reaction is highly dependent on pH (the signal falls *in vitro* by 40% between pH 7.2 and 6.8) [7,8], and unfortunately the intracellular pH (pH_i) changes markedly in cardiomyocytes during metabolic inhibition [9], which can lead to erroneous calculation of $[\text{ATP}]_i$ levels. The aim of this study was to eliminate these pH_i changes to obtain a more precise calibration of the luciferase signals.

Using ratiometric microfluorimetry of single cardiomyocytes loaded with the pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), we show that in Hepes-buffered media, poisoning of cells with CN^- and 2-deoxyglucose (2-DOG) leads to a rise and then a fall in pH_i . These changes in pH_i are abolished in medium buffered with 20% CO_2 /92 mM NaHCO_3 . We have also found that the presence of high concentrations of BSA, comparable to the protein concentration in cytoplasm, not only depresses the signal from luciferase at high ATP concentrations [10] but also raises the K_m (ATP) from approx. 400 μM [7] to approx. 2 mM. Using these refinements of the single-cell luciferase technique we show that rigor-induced consumption of cytoplasmic ATP in metabolically poisoned single cardiomyocytes consumes between 1 and 2 mM ATP in about 30 s, simultaneously with rigor-mediated cell shortening.

MATERIALS AND METHODS

D-Luciferin, firefly luciferase (from *Photinus pyralis*) and 2-DOG were purchased from Sigma. NaCN and the chemicals for perfusion media were obtained from Merck-BDM.

Abbreviations used: $[\text{ATP}]_i$, cytoplasmic ATP concentration; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; 2-DOG, 2-deoxyglucose; PCr, phosphocreatine; pH_i , intracellular pH.

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Adult Wistar rats (either sex) were anaesthetized with diethyl ether and killed by cervical dislocation. Ventricular cardiomyocytes were prepared by collagenase perfusion [11].

Measurements of pH_i

Cells were incubated for 30 minutes at room temperature in Hepes-buffered Krebs saline (in mM: NaCl, 125; KCl, 2.6; KH₂PO₄, 1.2; MgSO₄·7H₂O, 1.2; Hepes, 10; CaCl₂, 1; Glucose, 10; pH 7.4) with 0.05 % (w/v) pluronic F-127 (Calbiochem), and containing 10 µM BCECF-AM. Cells were then centrifuged gently, the supernatant was discarded and the pellet resuspended in Hepes-buffered Krebs saline to remove extracellular dye. A drop containing BCECF-loaded myocytes was placed in a trough machined in a stainless steel perfusion chamber, the base of which was a 22 mm round coverslip, and the cells were allowed to attach for 5 min. Inlet and outlet tubes connected to the chamber, which was maintained at a constant temperature of 37 °C, allowed continuous perfusion of the cells. After 5 min of perfusion with experimental medium to remove any remaining extracellular BCECF-AM, a myocyte was selected with a healthy rod-shaped appearance and regularly spaced sarcomeres. The pH_i was measured by dual-excitation of single BCECF-loaded cells, with a photon-counting microfluorometer (Cairn Research Limited, Sittingbourne, U.K.) in which the cell was illuminated 96 times a second, with excitation light at two wavelengths, 490 and 440 nm. The peak emission, at 535 nm, was recorded after the light had been passed through a long-pass filter at 530 nm. Calibration of the intracellular BCECF signal was performed *in situ* at the end of each experiment by using nigericin (5 µM) to equilibrate the intracellular and extracellular pH, by superfusing the cell with calibration solutions (in mM: Hepes, 10; Mes, 10; KCl, 140; EGTA, 1; MgSO₄, 1.2; KH₂PO₄, 1.2) adjusted in turn to several pH values (6.66, 7.02, 7.25, 7.53). The fluorescence ratios 490 nm/440 nm were linearly related to pH.

Clamping pH_i

Intracellular pH was clamped with a NaHCO₃-enriched Ringer solution equilibrated with a high CO₂ concentration (20 %). The NaHCO₃-buffered solution, pH 7.4 (in mM: NaCl, 33; KCl, 2.6; KH₂PO₄, 1.2; MgSO₄·7H₂O, 1.2; NaHCO₃, 92), was gassed with CO₂/O₂ (1:4) at 37 °C for 30 min before use. Liquid paraffin (BDH, heavy grade) was layered over solutions to prevent loss of CO₂ with consequent pH changes during the experiments. CO₂/O₂ (1:4) was blown into a covered area above the microscope perfusion chamber to maintain the partial pressure of CO₂. Determination of the perfusate pH at the end of each experiment showed that the pH remained constant during the experimental period.

Luciferase measurements

The technique for using firefly luciferase was essentially identical with that described previously [5,6] and involved microinjecting the enzyme into a cardiomyocyte and providing the luciferin in the perfusion medium at a concentration of 150 µM. Photon-counting instrumentation was used to detect the light emitted from a single injected cell while near-IR laser video microscopy gave simultaneous images of the cell.

Calibration of firefly luciferase *in vitro*

The firefly luciferase signal was calibrated *in vitro* with a luminometer (BioOrbit-1250), sensitive to 1.0 fmol of ATP. A 700 µl sample of a calibration solution designed to resemble the ionic constituents of cytoplasm (in mM: potassium gluconate,

110; KCl, 30; MgSO₄·7H₂O, 10; Hepes, 10; pH 7.07), and containing 180 mg/ml fatty acid-free BSA (Sigma, type A6003), to simulate cytoplasmic protein content [12], supplemented with the chosen ATP concentration, was placed into the luminometer cuvette at 37 °C and the background signal measured. Calibration solution containing luciferin was then added to give a total volume of 1 ml (luciferin final concentration, 15 µM) and the signal recorded. This process was repeated, varying the MgATP²⁻ concentration (0.1–8 mM).

Calibration of firefly luciferase in cytoplasm

Populations of (2–8) × 10⁶ mouse erythroleukaemia cells (MEL cells) stably transfected with the gene for *Photinus pyralis* firefly luciferase (a gift from Dr. E. Conley) were suspended in 2 ml of Dulbecco's minimal essential medium supplemented with luciferin (1 mM) and placed at 37 °C in the luminometer (BioOrbit-1250). Cells were poisoned with 2-DOG (15 mM) and the signal was allowed to decay to various levels over a period of 40 min. 2-DOG (15 mM) was chosen as it causes a decrease in bioluminescence intensity (51 ± 4 %) and in total ATP concentration (81 ± 6 %) without changing cell viability as assessed by Trypan Blue exclusion. At a chosen signal level the cell population was sampled (0.1 ml) and lysed into a conventional ATP assay *in vitro* (e.g. 0.9 ml of luciferase/luciferin cocktail, containing 0.3 mM luciferin and 0.1 % saponin). In this way the luciferase signals from intact cells could be related to a measured ATP content, allowing K_m (ATP) in cytoplasm to be estimated by using the Michaelis–Menten equation.

Calibration of firefly luciferase in *Escherichia coli*

E. coli (strain LE 392) was transfected with the gene for *Luciola mingrelica* luciferase with the pJCR plasmid (the plasmid was constructed in co-operation with the laboratory of Professor T. O. Baldwin, University of Houston, Houston, TX, U.S.A.) for overexpression of luciferase [13]. The cells were grown in Luria–Bertani medium containing 100 µg/ml ampicillin at room temperature to avoid the inactivation of luciferase by heat. Cells were washed and resuspended in experimental medium (in mM: Hepes, 10; NaCl, 150; KCl, 0.6) and 0.2 % glucose, pH 7.2. The time course of bioluminescence in *E. coli* cells was recorded in a cuvette in a BioOrbit-1250 luminometer. Cell suspension (10⁶ cells) was placed in a cuvette containing 1 ml of medium (defined above) containing 0.5 mM luciferin, polymyxin (6 × 10⁻⁵ units per cell) and different concentrations of MgATP²⁻. We checked that polymyxin in such concentration increases the permeability of *E. coli* membrane without killing the cells or releasing luciferase into the medium. The equilibration of extracellular ATP with polymyxin-permeabilized cells was shown by comparing the luciferase signal from intact cells (in which [ATP] is about 5 mM) with that obtained by adding 5 mM ATP to cells permeabilized with polymyxin for 1 h, after which the endogenous [ATP] and the luminescent signal had decayed to zero. The luciferase signals from the permeable *E. coli* cells at different concentrations of MgATP²⁻ were recorded and K_m (ATP) was estimated.

RESULTS

Measurements of pH_i in cardiomyocytes

Cardiomyocytes perfused with Hepes-buffered medium and poisoned with 2 mM CN⁻ and 5 mM 2-DOG undergo an initial increase in pH_i over 3–4 min from a resting value of 7.19 ± 0.04 (mean ± S.D.; n = 8) to 7.46 ± 0.1 (mean ± S.D.; n = 8), followed by a longer acidosis that slowed and then stabilized at pH_i 6.94 ± 0.1 (mean ± S.D.; n = 8) at the time of cell shortening.

Figure 1 Changes in pH_i in single cardiomyocytes

Upper panel: pH_i during metabolic inhibition in a BCECF-loaded cardiomyocyte perfused with Hepes-buffered solution. Poisoning with 2 mM CN^- and 5 mM 2-DOG starts at time zero. Lower panel: pH_i in a cardiomyocyte in $NaHCO_3$ -buffered solution (92 mM $NaHCO_3$ /20% CO_2), showing only very small pH_i changes during metabolic inhibition. In both panels S indicates onset of contracture.

Figure 1 (upper panel) shows such pH_i changes in a representative myocyte. In myocytes in 20% CO_2 / $NaHCO_3$ -buffered medium the resting pH_i was slightly lower, at 7.08 ± 0.07 (mean $\pm$ S.D.; $n = 8$), and poisoning induced very little pH_i change, such that at the time of shortening the pH_i was 7.07 ± 0.01 (mean $\pm$ S.D.; $n = 8$) (Figure 1, lower panel). Note that at the time the cells shortened (arrowed in Figure 1) there was no detectable pH_i change. There was a significant decrease (Student's t test, $P < 0.01$) in the time at which shortening occurred in the cells in which the pH_i was clamped (13.5 ± 4.6 min; mean $\pm$ S.D.; $n = 12$) compared with those in which acidosis had not been abolished (22.7 ± 7 min; mean $\pm$ S.D.; $n = 17$).

Firefly luciferase signals in cardiomyocytes

Figure 2 (upper panel) shows the response of the firefly luciferase signal from a single injected cardiomyocyte perfused with Hepes-buffered solution during metabolic inhibition with cyanide and 2-DOG, and during simulated reoxygenation (removal of cyanide). Shortly after the imposition of metabolic inhibition (1 min) there is an initial rise in luminescence, followed by a biphasic drop in the signal that is initially gradual, and then sudden at 12 min, after which the luciferase signal is barely detectable above background. All the cells subjected to this protocol ($n = 7$) followed the same pattern. In four out of seven cells, 2–3 min after removal of CN^- and 2-DOG from the perfusate and resupply of glucose a prompt rise in signal occurred from the background level to luminescence intensities at least

Figure 2 Luciferase signals from single cardiomyocytes

Upper panel: the characteristic response of the firefly luciferase signal in photoelectron counts per second (cps) from a myocyte during metabolic inhibition with CN^- and 2-DOG (starting at time zero) in Hepes-buffered solution. Removal of metabolic inhibition induces a partial recovery in luminescence, preceded by a large transient spike in the signal. Lower panel: the luciferase signal during and after metabolic inhibition from a myocyte perfused with bicarbonate-buffered solution, 92 mM $NaHCO_3$ /20% CO_2 . For both panels, 150 μ M luciferin was present in the perfusate throughout the experiment and photomultiplier background (1–3 c.p.s.) has been subtracted from the signals.

double the initial signal before poisoning. The luminescence then returned within 1–3 min to a level similar to or below the resting value, where it remained stable. In the remaining three out of seven cells signal recovery did not occur, probably owing to rupture of the cell membrane before the removal of metabolic inhibition, allowing the firefly luciferase to leak from the cell. These results closely resemble those described previously [5].

The response of the luciferase signal from single cardiomyocytes perfused with 20% CO_2 / $NaHCO_3$ -buffered media (Figure 2, lower panel; representative of $n = 7$), differed from that observed in myocytes in Hepes-buffered solution (Figure 2, upper panel) in that the initial rise in signal after poisoning did not occur. The subsequent slow fall in luminescence was also diminished; hence the final abrupt fall to a signal close to zero was more pronounced in these myocytes. There was no discernible difference in pattern of the luciferase signal during simulated reoxygenation from that in cells in which pH_i had not been clamped.

Simultaneous measurements of the luciferase signal and cell length by near-IR video microscopy [6] showed that myocyte shortening (to $47\% \pm 6.6$ the original length; mean $\pm$ S.D.; $n = 6$)

Figure 3 Simultaneous measurements of luciferase signal and cell length

The firefly luciferase signal from a pH-clamped cardiomyocyte (upper panel) monitored simultaneously with cell length (lower panel) during metabolic inhibition and simulated reoxygenation. Outlines of the shape of the cell, taken from the video recording, are also shown at selected time points in the lower panel.

under metabolic inhibition is synchronous with the sudden fall in the luminescence signal, and that rounding-up of the cell after reoxygenation coincides with the initiation of signal recovery (Figure 3).

Calibration of intracellular luciferase signals

Because previous results showed that the presence of high protein concentration greatly depressed the luminescence signal [10], we investigated the effect on the K_m (ATP) of the luciferase reaction. This value is important for attempting calibration of the intracellular luciferase signal, which is based on a knowledge of the cytoplasmic ATP content of the healthy cell and the relationship between the signal and ATP concentration *in vitro* [5,7]. Accordingly we have measured the K_m (ATP) *in vitro* in high concentrations of BSA that simulate the protein content of cytoplasm of mammalian cells [12]. We have also related the signal from intracellular enzyme to ATP content in luciferase-transfected mammalian cells during poisoning, and in luciferase-expressing *E. coli* in which intracellular enzyme is exposed to known ATP concentrations by using polymyxin to render the cells permeable to ATP.

K_m (ATP) for firefly luciferase at high protein concentrations

Figure 4(A) shows a dose-response curve of luciferase signal at different [ATP] in the presence of 180 mg/ml BSA, to simulate cytoplasmic protein levels, plotted as percentages of the signal at 7 mM, the resting [ATP]_i in a healthy myocyte. K_m (ATP) was 2 ± 0.3 mM, as estimated from the Michaelis-Menten relationship. The curve fit is a Sigma plot (nonlinear regression).

Figure 4 Relationship between luciferase signal and ATP concentration

(A) The dose-response curve of the luciferase light signal at various MgATP²⁻ concentrations *in vitro*. The calibration solution contained 180 mg/ml BSA and 15 μ M luciferin, pH 7.07. The signals were plotted as percentages of the signal detected at 7 mM ATP, the resting [ATP]_i in a healthy cardiomyocyte. (B) The relationship between luminescence from luciferase-transfected MEL cells with ATP measured by classical assay, in cells poisoned and then sampled at various levels of signal. (C) The dependence of the bioluminescent signal from polymyxin-permeabilized *E. coli* cells expressing firefly luciferase on the ATP concentration. Different concentrations of ATP were added to the suspension of cells in 10 mM Hepes, pH 7.2, containing 150 mM NaCl, 0.2% glucose, 0.6 mM KCl, polymyxin (6×10^{-5} unit per cell) and 0.5 mM luciferin.

K_m (ATP) for firefly luciferase in the cytoplasm of mammalian cells

Figure 4(B) shows the dependence of bioluminescent signal from a suspension of intact MEL cells poisoned with 15 mM 2-DOG on [ATP]_i as measured by a conventional ATP assay. In healthy cells the ATP content was 5 ± 2 mM (mean $\pm$ S.D., $n = 11$) and was not influenced by cell density within the range used. The dependence of bioluminescence intensity on intracellular [ATP]_i has been approximated by the Michaelis-Menten equation. The curve fit is a Sigma plot (nonlinear regression). The value for K_m (ATP), 1.6 ± 0.4 mM (mean $\pm$ S.D.; $n = 7$), is higher than that *in vitro* (0.4 mM). The K_m for luciferin with MEL cells was higher than in solution (170 ± 60 μ M compared with approx. 10 μ M).

Figure 5 Luciferase signals from single cardiomyocytes calibrated as ATP concentration

Calibration of the luciferase signal in two myocytes, both with pH_i clamped and subjected to poisoning and simulated reoxygenation. The calibration curve depicted in Figure 4(A) has been used to calibrate the luciferase signals, assuming that healthy myocytes contain 7 mM cytoplasmic ATP (see the text).

K_m (ATP) for firefly luciferase in *E. coli*

Figure 4(C) shows the dependence of the luminescent signal from polymyxin-permeabilized *E. coli* cells equilibrated with known ATP concentrations; K_m (ATP) was 4.6 ± 0.4 mM (mean $\pm$ S.D.; $n = 10$). Curve fit with hyperbolic regression: $a = (91.5 + 3.5)$ arbitrary units and $b = (4.6 + 0.4)$ mM. The K_m for luciferin for firefly luciferase inside *E. coli* cells was $40 \mu\text{M}$, similar to that in solution (approx. $10 \mu\text{M}$).

Calibration of cardiomyocyte signals

The values for K_m (ATP) *in vitro* in a high-protein milieu, in MEL cells and in *E. coli* were respectively 2, 1.6 and 4.6 mM. These are considerably higher than the K_m (ATP) of 0.4 mM described *in vitro* in the absence of protein but in similar mammalian ionic milieu at 37°C [7]. We have used the results obtained with a high BSA concentration (Figure 4A), because of the rather better defined conditions compared with the results from MEL cells and *E. coli*, to calibrate the single-cell luciferase signals of Figure 5, in which pH_i was clamped, in terms of $[\text{ATP}]_i$. The initial signal in the healthy cell was assumed to be generated by an $[\text{ATP}]_i$ of 7 mM, which is an average value derived from 11 published measurements ranging from 4.8 to 11.2 mM [14–24]. The right-hand ordinate of Figure 5 depicts $[\text{ATP}]_i$. Results from seven such experiments show consistently that the sudden fall in the luciferase signal started when the $[\text{ATP}]_i$ levels were between 2 and 3 mM ($n = 7$), and the onset of cell shortening occurred when $[\text{ATP}]_i$ was between 1 and 2 mM ($n = 7$). During shortening $[\text{ATP}]_i$ fell rapidly to $20 \pm 13.96 \mu\text{M}$ (mean $\pm$ S.D.; $n = 7$) in about 30 s. When simulated reoxygenation led to recovery of the signal, hypercontracture of the cell started when the $[\text{ATP}]_i$ was between 100 and $500 \mu\text{M}$ ($n = 3$) and the signals stabilized finally at a level corresponding to $[\text{ATP}]_i = 2\text{--}3$ mM ($n = 7$).

DISCUSSION

Our results with the fluorescent probe BCECF showed that in cells in Hepes-buffered medium an initial alkalosis occurs during metabolic inhibition (Figure 1, upper panel), probably because of a net consumption of protons by phosphocreatine (PCr) breakdown. This is followed by a longer acidosis, due mainly to H^+ production during ATP hydrolysis [9,25]. Thus a total pH_i variation of 0.52 pH units occurs. This is sufficient to create serious pH-dependent artifacts in the luciferase signal that interfered with previous measurements of $[\text{ATP}]_i$ with firefly luciferase [5,6]. Indeed, Koop and Cobbold [7] showed that at an ATP concentration of 5 mM *in vitro*, the luciferase signal at pH 6.8 was 35% lower than at pH 7.2. Perfusion of myocytes with medium buffered with 92 mM $\text{NaHCO}_3/20\%$ CO_2 was almost completely effective in clamping pH_i throughout chemical hypoxia (Figure 1, lower panel). Bicarbonate greatly enhances the internal buffering capacity of myocardial cells, increasing their resistance to changes in pH_i [26]. In bicarbonate-buffered medium, in addition to the Na^+/H^+ exchanger, a voltage-insensitive $\text{Na}^+/\text{HCO}_3^-$ symport carrier is the most important mechanism of acid extrusion, whereas a $\text{Cl}^-/\text{HCO}_3^-$ counter-transport acts in response to intracellular alkalosis [27,28]. The onset of cell shortening was delayed in cells in which intracellular acidosis had not been abolished during metabolic inhibition. Similar findings have been previously reported [9,29] and have been attributed to a decrease in energy demand by mild acidosis, independent of the negative inotropic effect of acidosis [30].

The luciferase luminescence from cardiomyocytes superfused with bicarbonate-buffered solution can be considered free of pH_i -dependent artifacts because the pH_i of these cells has been shown to be completely clamped, so variations in the signal can more confidently be attributed to changes in $[\text{ATP}]_i$. The initial rise in the luciferase signal that occurred in the myocytes in Hepes-buffered medium after application of CN^- and 2-DOG is not observed in the cells in which the pH_i has been clamped (Figure 2). This confirms that the increase in signal was indeed likely to be caused by consumption of H^+ during PCr breakdown. The subsequent slow decrease in signal seems to be slightly diminished, resulting in a final sudden fall from higher ATP levels than in Hepes-buffered cells. The abrupt fall in luciferase signal to background levels still occurs in cells in bicarbonate-buffered media simultaneously with the onset of cell contracture, as illustrated in Figure 3. This observation endorses the working hypothesis [4] that predicts a rapid consumption of cytoplasmic ATP triggered by formation of rigor-complexes, causing cell shortening.

Calibration of the cytoplasmic luciferase signal in terms of ATP concentration is dramatically affected by correcting for the effect of high concentrations of protein, a fact that was not known in our earlier work [5,6]. The luciferase K_m for ATP was shifted 10-fold higher in the presence of BSA in the medium. Firefly luciferase is a euglobulin that is thought to function as a dimer [31]. We postulate that the presence of competing hydrophobic sites on BSA, or on cytoplasmic proteins, affects the kinetics of dimerization of luciferase and hence both the luminescence intensity at saturating ATP [10] and the K_m (ATP) (Figure 4A), which is about 10 times that described for the same ionic milieu, chloride concentrations and temperature [7]. A K_m (ATP) value comparable with that in high BSA concentration *in vitro* was found by using luciferase-transfected MEL cells (Figure 4B) and *E. coli* (Figure 4C).

The calibration curve obtained with high concentrations of BSA (Figure 4A) has been applied to the signal from myocytes with pH_i clamped. The estimated $[\text{ATP}]_i$ at which cell shortening

starts was always between 1 and 2 mM. These values in the millimolar range are considerably higher than the 150 μ M calculated by Bowers et al. [5] for the start of cell shortening. The effect of high protein concentration or cytoplasm on the K_m (ATP) of luciferase was not known in that earlier study; this recalibration accounts for much of this difference. Furthermore Bowers et al. [5] normalized the luciferase signal from the healthy cell as 5 mM ATP. However, a more accurate value for resting $[ATP]_i$ in healthy cardiomyocytes is 7 mM, based on pooled results from 11 studies [14–24]. Accordingly in Figure 5 the signal from the healthy cell at the start of the experiment has been normalized to 7 mM ATP (right-hand ordinate). In addition to the differences that can be accounted for by differences in the signal calibration, clamping the pH_i in cells might also lead to contracture at higher $[ATP]_i$. These myocytes did not undergo acidification, and it has been observed that the critical $[ATP]$ necessary for inducing rigor contraction from the relaxed state is decreased with decreasing pH [32,33]. However, the difference in pH_i at the onset of rigor between our Hepes-buffered and HCO_3^-/CO_2 -clamped cells was only 0.13 pH unit, so the predicted percentage change in the ATP concentration needed to induce rigor would be only 20% [32,33]. An ATP concentration of 1–2 mM for the start of cell shortening is in agreement with published levels of ATP needed for the onset of rigor tension development in isolated ferret hearts, and rat skinned myocytes in the absence of PCr [22,34–36]. Rapid depletion of PCr during anoxia would be the main factor responsible for the high threshold $[ATP]_i$ in the millimolar range, for the development of rigor tension observed here because, as shown by Ventura-Clapier and Veksler [33], a decrease in [PCr] shifts rigor tension development towards higher [ATP]. Indeed, Ventura-Clapier and Veksler [33] showed that in rat ventricular skinned fibres in the absence of PCr, rigor contracture is initiated at 1 mM ATP, whereas in 12 mM PCr it starts at 100 μ M ATP.

Our results indicate that as a cell shortens it consumes between 1 and 2 mM ATP in about 30 s, which would be expected to induce a pronounced decrease in pH_i . However, no acceleration of the decrease in pH_i occurs during shortening (Figure 1). We presume that the greatly elevated inorganic phosphate concentration, which is commensurate with the loss of PCr (25 mM [14]), provides sufficient buffering capacity to prevent a decrease in pH_i . Indeed, the pK_a for the $H^+ + HPO_4^{2-} \rightleftharpoons H_2PO_4^-$ buffer system is 6.8, close to the lowest pH_i (6.9) obtained in cells in Hepes medium (Figure 1, upper panel).

The recovery of $[ATP]_i$ after simulated reoxygenation (to 2–3 mM) is also greater than previously reported by Bowers et al. [6] (200–450 μ M), although it is still incomplete, possibly owing to loss of adenosine. This ATP recovery is preceded by a transient rise in signal to a level considerably higher than the initial signal in the healthy cell. It is most unlikely that this is caused by an overshoot in ATP concentration, nor can it be due to a transient increase in pH_i because pH_i measured with BCECF remained constant (results not shown). It is known that the addition of ATP to a mixture of luciferase, luciferin and Mg^{2+} produces a flash of light followed by a rapid decay of light emission [37]. This situation might be equivalent to the cytoplasmic conditions in a myocyte after removal of metabolic blockade. ATP would be resynthesized and would mix with luciferase and luciferin already present in the cytosol, generating a peak of light. However, *in vitro* the light peak ('flash') has a duration of seconds, whereas the myocyte signals are of approx. 2 min duration. This delay could be caused by the peculiar conditions in the cytosol that are not reproduced *in vitro*; the effect of high protein concentration on the flash kinetics requires investigation *in vitro*. The luminescence of firefly luciferase is also

influenced by other cytoplasmic constituents such as CoA and PP_i , both of which can enhance luminescence at a given ATP concentration. Higher concentrations of PP_i (above 50 μ M) are inhibitory [38]. Because we do not know the time course of any change in CoA or PP_i in the cytoplasmic compartment of these cells, their possible contributions to the complex pattern of the luciferase signal cannot be assessed.

The free energy of ATP hydrolysis (ΔG_p), calculated from $[ATP] = 1.5$ mM (see above), $[ADP] = 100$ μ M [39] and $[P_i] = 25$ mM [14], is -47 kJ/mol at the time of onset of cell shortening. Although this value is below the ΔG_p calculated for healthy tissue, it is well above the energy requirements of the various ion pumps: 43 kJ/mol for SR Ca pump, 44 kJ/mol for Na,K-ATPase, and 39 kJ/mol for the plasmalemmal Ca,Mg-ATPase [40]. By the time the cell has shortened completely, $[ATP]_i$ is about 20 μ M (see above) and the ΔG_p has fallen to -36 kJ/mol, which is insufficient to maintain ionic homeostasis by the ion-transport ATPases. Furthermore $[ATP]_i$ has fallen to around 10% of the K_m (ATP) for the sodium pump, and close to the K_m (ATP) for the plasmalemmal Ca^{2+} pump. Thus ATP depletion, in conjunction with elevated $[P_i]$, is a plausible explanation for the loss of ionic homeostasis observed predominantly after cell shortening [2,3,41].

In conclusion, eliminating changes in pH_i during metabolic inhibition in single cardiomyocytes, together with a calibration protocol that embraces the effect of protein in the cytoplasm on luciferase K_m (ATP), provides a more accurate calibration of firefly luciferase signals in terms of $[ATP]_i$. The synchronism between cell shortening and a precipitous depletion of cytoplasmic ATP (from 1–2 mM to approx. 20 μ M), observed during metabolic poisoning, endorses our working hypothesis that rigor activation of myosin S1-ATPase depletes the cardiomyocytes of ATP and that this precipitates ion-pumping failure. The new calibration of the luciferase signal shows that the rigor-precipitated $[ATP]_i$ decrease is considerably greater than previously estimated, consuming 15–30% of the ATP content of a healthy cell in approx. 30 s and decreasing $[ATP]_i$ to approx. 0.3% of its level in the healthy cell. The hypercontracture observed on reoxygenation would be due to a rapid resupply of cytoplasmic ATP along with pre-existent elevated $[Ca^{2+}]_i$. We conclude that rigor-activation of myosin ATPase should be regarded as a key target for pharmacological intervention.

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