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

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INTRODUCTION

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 (pHi) can be clamped at pH 7.08 by perfusing cells with a modified bicarbonate-buffered Krebs saline containing 92 mM NaHCO3 and equilibrated with 20% CO2. The signal from microinjected firefly luciferase was therefore developed [5].

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 Ca2+ concentration ([Ca2+]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 [Ca2+]i in cardiomyocytes subjected to metabolic poisons or anoxia follows [Ca2+]i-independent, rigor-mediated cell shortening [1–3]. To explain the relation between the sudden shortening of metabolically poisoned cardiomyocytes and the loss of control of calcium homeostasis, we show that in Hepes-buffered media, poisoning of cells with CN− and 2-deoxyglucose (2-DG) leads to a rise and then a fall in pH. These changes in pH, changes markedly in cardiomyocytes during metabolic inhibition [9], which can lead to erroneous calculation of [ATP], levels. The aim of this study was to eliminate these pH changes to obtain a more precise calibration of the luciferase signals.

INTRODUCTION

Cytoplasmic ATP concentration ([ATP]) can be clamped at 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 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.
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**

Cells were incubated for 30 minutes at room temperature in Hepes-buffered Krebs saline (in mM: NaCl, 125; KCl, 2.6; KH$_2$PO$_4$, 1.2; MgSO$_4$, 7H$_2$O, 1.2; HEPES, 10; CaCl$_2$, 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 perfusion chamber to maintain the partial pressure of CO$_2$.

**Calibration of firefly luciferase in cytoplasm**

E. coli (strain LE 392) was transfected with the gene for *Luciola mingrelica* luciferase with the pJR 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 (10$^6$ 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 MgATP$^2^\text{--}$. 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 calibration 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 MgATP$^2^\text{--}$ were recorded and $K_m$ (ATP) was estimated.

**RESULTS**

**Measurements of pH in cardiomyocytes**

Cardiomyocytes perfused with HEPES-buffered medium and poisoned with 2 mM CN$^\text{--}$ and 5 mM 2-DOG undergo an initial increase in pH 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 6.94 ± 0.1 (mean ± S.D.; n = 8) at the time of cell shortening.
Rigor and ATP in cardiomyocytes

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 ± 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 ± 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 ± S.D.; n = 12) compared with those in which acidosis had not been abolished (22.7 ± 7 min; mean ± 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 the firefly luciferase leaked 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 spike (Figure 2, upper panel) at 12 min was less 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% ± 6.6 the original length; mean ± S.D.; n = 6)
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], in a healthy myocyte. $K_m$ (ATP) was $2 \pm 0.3$ mM, as estimated from the Michaelis–Menten relationship. The curve fit is a Sigma plot (nonlinear regression).

### $K_m$ (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], as measured by a conventional ATP assay. In healthy cells the ATP content was $5 \pm 2$ mM (mean ± S.D., $n = 11$) and was not influenced by cell density within the range used. The dependence of bioluminescence intensity on intracellular [ATP] has been approximated by the Michaelis–Menten equation. The curve fit is a Sigma plot (nonlinear regression). The value for $K_m$ (ATP), $1.6 \pm 0.4$ mM (mean ± 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 ($170 \pm 60$ µM compared with approx. 10 µM).
**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 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 [ATP], 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 NaHCO₃/20% CO₂ was almost completely effective in clamping pH through chemical hypoxia (Figure 1, lower panel). Bicarbonate greatly enhances the internal buffering capacity of myocardial cells, increasing their resistance to changes in pH [26]. In bicarbonate-buffered medium, in addition to the Na⁺/H⁺ exchanger, a voltage-insensitive Na⁺/HCO₃⁻ symport carrier is the most important mechanism of acid extrusion, whereas a Cl⁻/HCO₃⁻ countertransport 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-dependent artifacts because the pH of these cells has been shown to be completely clamped, so variations in the signal can more confidently be attributed to changes in [ATP]. 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 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 Km 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 kinetics of dimerization of luciferase and hence both the luciferase signal and the ATP concentration at which luciferase signals are generated by an ATP calibration curve. In Figure 4A, the calibration curve obtained with high concentrations of BSA (Figure 4A) has been applied to the signal from myocytes, assuming that healthy myocytes contain 7 mM cytoplasmic ATP (see the text).

**Kₘ (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; Kₘ (ATP) was 4.6 ± 0.4 mM (mean ± S.D.; n = 10). Curve fit with hyperbolic regression: \( a = (91.5 ± 3.5) \) arbitrary units and \( b = (4.6 ± 0.4) \) mM. The Kₘ for luciferin for firefly luciferase inside E. coli cells was 40 µM, similar to that in solution (approx. 10 µM).

**Calibration of cardiomyocyte signals**

The values for Kₘ (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 Kₘ (ATP) in a high-protein milieu, in mammalian ionic milieu at 37 °C [7]. A Kₘ (ATP) value comparable with that in high BSA concentration media has been found in about 30 s. When simulated reoxygenation led to recovery of the signal, hypercontracture of the cell started when the [ATP], was between 100 and 500 µM (n = 3) and the signals stabilized finally at a level corresponding to [ATP], = 2–3 mM (n = 7).
starts was always between 1 and 2 mM. These values in the millimolar range are considerably higher than the 150 μM 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], 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 in cells might also lead to contracture at higher [ATP]. 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 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 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], 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 μM 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. However, no acceleration of the decrease in pH 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. Indeed, the pK$_a$ for the H$^+$–HPO$_4^{2-}$ buffer system is 6.8, close to the lowest pH (6.9) obtained in cells in Hepes medium (Figure 1, upper panel).

The recovery of [ATP], after simulated reoxygenation (to 2–3 mM) is also greater than previously reported by Bowers et al. [6] (200–450 μM), 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 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 μM) 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 ($\Delta G_c$), calculated from [ATP] = 1.5 mM (see above), [ADP] = 100 μM [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 $\Delta G_c$ 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$_{2+}$ pump [40]. By the time the cell has shortened completely, [ATP], is about 20 μM (see above) and the $\Delta G_c$ has fallen to −36 kJ/mol, which is insufficient to maintain ionic homeostasis by the ion-transport ATPases. Furthermore [ATP], 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 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]. The synchronism between cell shortening and a precipitous depletion of cytoplasmic ATP (from 1–2 mM to approx. 20 μM), observed during metabolic poisoning, endorses our working hypothesis that rigor activation of myosin S1-ATPase depletes the cardiomyocytes of ATPase, and 39 kJ/mol 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].

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Rigor and ATP in cardiomyocytes


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