Continuous bioluminescent monitoring of cytoplasmic ATP in single isolated rat hepatocytes during metabolic poisoning

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We have devised a technique for monitoring cytoplasmic ATP continuously in single hepatocytes. Single isolated rat hepatocytes were injected with the ATP-dependent luminescent protein firefly luciferase, and then superfused with 45 μ M luciferin in air-equilibrated medium. Signals of approx. 10–200 photoelectron counts per second could be recorded from individual healthy cells for up to 3 h. The response of the luminescent signal to chemical hypoxia (2–5 mM CN^- and 5–10 mM 2-deoxyglucose) was monitored. We found a great cell-to-cell variability in the time course of the ATP decline in response to CN^- , 2-deoxyglucose or to their combination; the time for the signal to fall to 10% of the original (corresponding to approx. 100 μ M ATP) ranged from approx. 20 to 75 min. This resistance of the

cytoplasmic ATP concentration to depletion after blockade of oxidative phosphorylation and glycolysis could be abolished by pretreatment of the cells with etomoxir, which blocks mitochondrial β -oxidation. Etomoxir alone had no effect on the luciferase signal, but etomoxir-pre-treated cells showed a prompt fall in the luciferase signal starting within 1–2 min of application of cyanide and 2-deoxyglucose and falling to 10% of the original signal in approx. 6–10 min. The technique allows cytoplasmic ATP changes to be monitored in single hepatocytes at concentrations of 1 mM or lower, but more precise calibration of the signal will require correction for the effects of cytoplasmic pH changes.

INTRODUCTION

Measurements in single cells have proved to be particularly informative in resolving the sequence of events occurring in cells exposed to pathological insults. The widely popular calcium hypothesis of cell death, in which a rise in cytoplasmic free Ca^{2+} was believed to occur relatively early in the response to metabolic poisoning, was rapidly refuted once single-cell measurements of free Ca^{2+} became feasible (Cobbold and Bourne, 1984; Lemasters et al., 1987). In hepatocytes, single-cell free- Ca^{2+} measurements using fura-2 showed convincingly that blebbing of the plasmalemma, a visible cellular response to hypoxia, could occur without any detectable rise in free Ca^{2+} (Lemasters et al., 1987). The stimulus for blebbing of the plasmalemma is not yet known, but is unlikely to be resolved from measurements on cell populations, because blebbing does not occur synchronously within cells in a population, and because blebs are dynamic reversible structures. The impetus for attempting to monitor cytoplasmic ATP in single cells came from the belief that metabolic poisoning must at some point result in a lowering of cytoplasmic ATP, and that this fall in ATP may also not be synchronous between cells. Using techniques developed for injecting and monitoring the luminescence of aequorin in single heart and liver cells (Cobbold and Lee, 1991), we have recently succeeded in using firefly luciferase to monitor sarcoplasmic ATP in single cardiomyocytes (Bowers et al., 1992). Here we describe the use of firefly luciferase to monitor cytoplasmic ATP in single isolated rat hepatocytes. Many attempts have been made to measure the cytoplasmic ATP concentration of isolated cells. Measurements by h.p.l.c. result only in total ATP concentrations in cell populations, as described for cardiomyocytes (Siegmund et al., 1990) and for hepatocytes (Anundi et al., 1987), and the same applies to n.m.r. methods. It is possible to measure

cytoplasmic ATP with the non-aqueous fractionation method (Elbers et al., 1974), but this method is also only feasible with cell suspensions, and is extremely complex. We report here a novel method for monitoring cytoplasmic ATP in single isolated hepatocytes. This method is based on the luminescence of firefly luciferase from *Photinus pyralis*, whose kinetics and properties have been extensively reviewed by DeLuca and McElroy (1974) and DeLuca (1976).

MATERIALS AND METHODS

D-Luciferin, firefly luciferase (from *Photinus pyralis*), 2-deoxyglucose and AICA-riboside (5-amino-4-imidazolecarboxamide riboside) were purchased from Sigma. NaCN and the chemicals for perfusion media were obtained from Merck-BDH. Etomoxir, as the racemic sodium salt (B827-33), was generously given by Dr. H. P. O. Wolf (Byk Gulden Chemische Fabrik, Konstanz, Germany).

Male Wistar rats (250–300 g) were killed by cervical dislocation, and the hepatocytes were prepared by the collagenase-perfusion method (Seglen, 1972). The isolated hepatocytes were incubated at 37 °C in Williams' medium E buffered with 5% CO_2 /bicarbonate in air for at least 1 h before they were set up for microinjection, supported in gelled agarose in flat glass capillaries, as described by Cobbold and Lee (1991).

Solutions of firefly luciferase (20 mg/ml) were made up in stock buffer (100 mM KCl, 1 M glycylglycine, 0.1% NaN_3 , pH 7.75) and stored at 4 °C. Before use, approx. 200 nl of this stock of luciferase was microdialysed (Cobbold and Lee, 1991) against dialysis buffer [30 mM KCl, 90 mM potassium gluconate, 30 mM KH_2PO_4 , 1 mM Pipes, 125 μ M EGTA, 1 mM dithiothreitol, 20 mg/ml polyvinylpyrrolidone (average M_r 40000), pH 7.25]. Micropipettes were filled with dialysed luciferase solu-

Abbreviations used: AICA-riboside, 5-amino-4-imidazolecarboxamide riboside; c.p.s., photoelectron counts per second.

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tion through the tip as previously described for aequorin (Cobbold and Lee, 1991).

After 30 min recovery from microinjection with luciferase solution to 1–5% of cell volume, a single healthy-looking cell was transferred in the microslide to a stainless-steel cup held at 37 °C under a cooled S20-trialkali photomultiplier (E.M.I. type 9863A/350) similar to that described by Cobbold and Lee (1991).

A hepatocyte was initially superfused with a glucose-free Ringer solution (116 mM NaCl, 5.6 mM KCl, 0.8 mM MgSO_4 , 1 mM NaH_2PO_4 , 1 mM KH_2PO_4 , 4.8 mM NaHCO_3 , 1.8 mM CaCl_2 , 20 mM Hepes, pH 7.2). Chemical hypoxia was then induced, after between 12 and 70 min, depending on the cell (see the Figure legends), by the same medium containing metabolic inhibitors. All media contained 45 μM luciferin (Bowers et al., 1992), added from a 10 mM stock dissolved in glucose-free buffer and stored at –70 °C in the dark.

Experiments *in vitro* were carried out with dialysed luciferase held as a thin film inside a short length of 160 μm -bore microdialysis tubing (Bio-Rad Biofiber 50A); the ends of the tube and most of the lumen were filled with liquid paraffin. To calibrate the luciferase light signal, the enzyme was superfused at 37 °C with injection buffer containing 45 μM luciferin at different pH values and containing a range of MgATP concentrations. As the luciferase reaction requires oxygen, this buffer and all other media were air-equilibrated. To test the effect of several metabolic inhibitors on firefly luciferase, the above medium contained saturating ATP concentrations (5 mM) and the respective inhibitor at its effective concentrations at pH 7.2.

RESULTS AND DISCUSSION

Signals between 10 and 200 photoelectron counts per second (c.p.s.) can be recorded from a healthy cell microinjected with luciferase and superfused with luciferin-containing medium. All the data presented here were obtained on cells maintained throughout the luciferase measurements in glucose-free Ringer

Figure 1 The light signal (in c.p.s.) recorded from a single hepatocyte microinjected with firefly luciferase and exposed to glucose-free Ringer's solution containing 45 μM luciferin

The box indicates where glucose (10 mM) was added to the superfusate. For the first 1 h the signal was reasonably stable at approx. 70 c.p.s. above the photomultiplier-generated background of 8 c.p.s. (bkgr).

solution, to ensure that subsequent inhibition of glycolysis with 2-deoxyglucose was as effective as possible. Figure 1 shows that the luciferase signal in such a cell was stable for approx. 1 h, and showed only a slow fall over the subsequent 1.5 h. Firefly luciferase can be post-translationally partitioned into peroxisomes, so absolutely stable signals over periods of more than 1 h or so would not be expected. The different initial signals in the healthy cells (Figures 1–4) arise from different activities in the luciferase samples, and differences in the volume injected, which was approx. 1–5% of cell volume. The effect of CN^- and 2-deoxyglucose on single isolated hepatocytes in glucose-free medium can be seen in four typical traces in Figure 2. The zero-time points in Figure 2 correspond to the onset of poisoning with the indicated concentrations of CN^- and 2-deoxyglucose. The light signal started to decrease sometime between 20 min (Figure 2a) and 63 min (Figure 2d). There was some experimental variation in the time at which inhibitors were added (and hence in the duration of exposure of the cell to glucose-free medium), but this does not appear to affect the time course of the decline in the signal. For instance, the time course in cells (a) and (b) (Figure 2) is similar, yet cell (a) had been exposed to zero-glucose medium for 70 min, and cell (b) for 18 min. In a total of eight cells, the fall of signal to a nominal end point (10% of initial signal) varied from 22 min to 75 min (mean $\pm$ S.D. = 44 ± 17 min). These differences in time course are unlikely to be due to the differences in either the CN^- or 2-deoxyglucose concentrations, nor in the period of exposure to glucose-free Ringer solution before adding poisons (compare Figures 2a and 2b, with glucose-free periods of 70 and 18 min respectively). The decline in signal is initially slow, but is followed by a rather steep decline to a signal close to zero. This steep decline in signal consistently occurred over $10 (\pm 2)$ min. The background level ('bkgr' in all these Figures) represents zero light emitted from the cell and is generated mainly by photoelectrons released thermally from the photocathode. The fall in the signal to near-zero levels is not caused by inactivation or loss of luciferase (see below).

Continuous Nomarsky microscopy of four groups of hepatocytes, each containing 3–5 cells, treated under the same conditions as the luciferase-injected cells with 4 mM CN^- and 10 mM 2-deoxyglucose, showed that the time course of plasmalemma blebbing was also variable from cell to cell, the first blebs appearing between 15 and 90 min after poisoning, independent of cell size (results not shown).

The results of metabolic inhibition with either CN^- alone or 2-deoxyglucose alone also show variability from cell to cell in the time course of the decline of the luciferase signal. The effect of CN^- alone (Figures 3a and 3b) is similar to the effect of the combination of CN^- and 2-deoxyglucose (Figure 2); the luciferase signal stayed constant for 35 (Figure 3a) and 60 min (Figure 3b), whereupon it fell to close to the background level. In a total of 6 cells exposed to CN^- alone (2–5 mM), the time to reach 10% of initial signal ranged from 10 to 65 min, with a mean of 38 ± 24 (S.D.) min. The rate of fall of the signal during the plunge in the signal usually appeared to be somewhat faster than with the combined inhibition by CN^- and 2-deoxyglucose (Figure 3).

When only glycolysis was blocked, the light signal decreased later than with CN^- alone. In Figure 3(c) the luciferase signal remained at initial levels until more than 60 min after poisoning with 5 mM 2-deoxyglucose alone (and a total of 100 min in glucose-free solution). For four cells the time to reach 10% of the initial signal ranged from 51 to 80 min, with a mean of 61 ± 11 min. Furthermore, the plunge in the signal appeared to be less steep than with the combined effect of CN^- and 2-

Figure 2 Light signals (in c.p.s.) recorded from single hepatocytes injected with firefly luciferase and superfused with glucose-free Ringer's solution containing $45 \mu\text{M}$ luciferin and metabolic poisons

Zero time denotes onset of chemical hypoxia by application of (a) 3 mM CN^- and $5 \text{ mM 2-deoxyglucose (DOG)}$, (b) 2 mM CN^- and 10 mM DOG , (c) 4 mM CN^- and 5 mM DOG , (d) 2 mM CN^- and 10 mM DOG . The times for which the cells were exposed to glucose-free Ringer's solution before zero time on these traces varied somewhat (a, 70 min; b, 18 min; c, 40 min; d, 16 min). 'bkgr' denotes the photomultiplier background count rate in the absence of light.

deoxyglucose (Figure 2), or indeed with CN^- alone (Figures 3a and 3b). Similar results were obtained by using, in addition to 2-deoxyglucose, two further inhibitors of glycolysis, fluoride and AICA-riboside (Figure 3d). AICA-riboside was recently shown to inhibit glycolysis (Vincent et al., 1992).

It was possible to restore the luciferase signal, and hence cytoplasmic ATP, even when the signal had fallen to near-background levels. Removing the metabolic inhibitors and superfusing the cell with aerated medium resulted in recovery of the light signal (Figure 3b). This recovery of the signal is good evidence that the luciferase has not been inactivated by the cell, nor leaked out of the cell. The transient peak shown after CN^- removal in Figure 3(b) (at around 75 min) was consistently found in many cells after removal of inhibitors. Cardiomyocytes subjected to this same experimental protocol show a peak signal that can exceed the signal in the healthy cell by 100%. This effect is not due to a large rise in intracellular pH, and its origin is not yet known in either cell type.

In liver, mitochondrial β -oxidation of fatty acids is an important source of acetyl-CoA, for sustaining the tricarboxylate cycle. Conceivably, β -oxidation could be responsible for main-

Figure 3 Light signals (in c.p.s.) from single hepatocytes superfused with glucose-free Ringer's solution containing $45 \mu\text{M}$ luciferin and metabolic poisons

Zero time denotes onset of metabolic blockade with: (a) 2 mM CN^- ; (b) 4 mM CN^- , and its removal; (c) $5 \text{ mM 2-deoxyglucose (DOG)}$; (d) inhibition of glycolysis with $50 \mu\text{M AICA-riboside}$, 5 mM F^- and 10 mM DOG , followed by 4 mM CN^- . The inhibitors were added after variable times in glucose-free Ringer's solution (a, 14 min; b, 21 min; c, 20 min; d, 12 min).

taining cytoplasmic ATP sufficient to saturate luciferase in the face of (presumably) complete blockade of glycolysis. Fatty acid β -oxidation can be blocked by etomoxir {2-[6-(4-chlorophenoxy)-hexyl]oxirane-2-carboxylate} (Selby and Sherratt, 1989). The *R*-enantiomer of etomoxir, after esterification by cytoplasmic acyl-CoA synthetase to etomoxir-CoA, inhibits carnitine palmitoyltransferase I ($\text{IC}_{50} 10 \text{ nM}$), thereby eliminating acyl-carnitine transport into the mitochondria. Figure 4 shows that pretreating hepatocytes with etomoxir ($1 \mu\text{M}$) for between 20 and 25 min has little effect on the luciferase signal; but when CN^- and 2-deoxyglucose are then applied, the luciferase signal starts to fall promptly, with little difference between cells in the time course. The time for the signal to fall to 10% of the initial level in the healthy cell was between 8 and 13 min (5 cells), considerably more rapid than in cells exposed to CN^- and 2-deoxyglucose but not pretreated with etomoxir (Figure 2). We cannot completely eliminate the possibility that the somewhat extended period in zero-glucose solution (41–55 minutes) underlies the prompt effect of CN^- and 2-deoxyglucose in these experiments. However, cells (a) and (c) of Figure 2 were exposed to glucose-free medium for 70 and 40 min respectively (a period comparable with that for the cells in Figure 4), and showed a

Figure 4 Firefly luciferase signals from three hepatocytes exposed first to etomoxir (Etx; 1 μ M), to block fatty acid β -oxidation, followed 20 min later by, in addition, CN^- (4 mM) and 2-deoxyglucose (DOG; 10 mM)

Photomultiplier background has been subtracted from these recordings. Zero time indicates when the cells were first exposed to zero-glucose medium and luciferin.

much delayed and slower decline in signal on addition of CN^- and 2-deoxyglucose.

If CN^- and 2-deoxyglucose induce prompt and complete blockade of both oxidative phosphorylation and glycolysis, and assuming that turnover of cytoplasmic ATP is rapid compared with the time course of these experiments, then other metabolic pathways are probably responsible for maintaining cytoplasmic ATP above 1 mM for 20–60 min (Figure 2). Hepatocytes possess high activity of adenylate kinase (Zhang and Kraus-Friedmann, 1989) but this pathway, which sacrifices two molecules of ADP to generate one of ATP, would be most unlikely to be able to maintain ATP levels for such prolonged periods. The most likely explanation is that the luciferase signal is being sustained by ATP generated from β -oxidation of fatty acids in the mitochondrial matrix, the cells utilizing endogenous stores of triacylglycerol since no metabolic substrates are present in the Ringer solution. We are unable to explain why β -oxidation should sustain the signal when the respiratory chain is blocked with CN^- (e.g. Figure 2), which binds to cytochrome oxidase with $K_d = 10^6$ – 10^4 M (Solomonson, 1981). Substrate-level phosphorylation in the tricarboxylate cycle, which generates one

Figure 5 Calibration *in vitro* of the luciferase light signal versus ATP concentrations, at four different values of pH at 37 °C in medium designed to simulate the ionic milieu of cytoplasm

The medium composition was potassium gluconate 110 mM, KCl 30 mM, KH_2PO_4 1 mM, MgSO_4 0.6 mM, Hepes 10 mM). The luciferase light signals were normalized to the signal given by the sample in 5 mM MgATP at pH 7.2 (100%). Curve-fit with simple hyperbolic regressions: variables a and b for pH 7.2 were 113.705 and 0.5, for pH 6.8 75.863 and 0.332, for pH 6.7 66.278 and 0.419 and for pH 6.3 51.536 and 0.449.

molecule of GTP per molecule of acetyl-CoA entering the cycle, may be important.

Iodoacetate is commonly used as an inhibitor of glycolysis (e.g. Lemasters et al., 1987). Iodoacetate (1 mM) promptly depresses the luciferase signal (results not shown), in marked contrast with the lack of effect of 2-deoxyglucose over much longer periods (e.g. Figure 3c). However, iodoacetate has a direct but reversible effect on firefly luciferase *in vitro* (results not shown), and is thus unsuitable for use in these experiments.

We have attempted to convert the luciferase light signal from photoelectron counts per second (c.p.s.) into cytoplasmic ATP concentrations. The determinations *in vitro* of the dependence of the luciferase signal on MgATP concentrations were performed with luciferase in micro-dialysis tubing at four different pH values and at ATP concentrations from 5 mM down to 0.2 mM and zero. The buffer was designed to mimic intracellular ionic concentration, particularly Cl^- , and the temperature was held at 37 °C. Despite a steady superfusion of fresh air-equilibrated medium containing ATP and 45 μ M luciferin, the luciferase signal decayed with a half-life of about 20 min, which contrasts with the remarkably stable intracellular signals (e.g. Figure 1). The reason for this is not yet known; we suspect thermal denaturation and destruction of the protein at the oil–water interface. During measurements *in vitro* in a series of MgATP concentrations it was therefore necessary to correct for this steady decline in luciferase activity by continually normalizing the signals by repeated application of 5 mM ATP.

Figure 5 shows the effect of MgATP concentration on the luciferase light signal after curve-fitting with the simple hyperbolic regression:

$$f(y) = \frac{ax}{(b+x)} \quad (1)$$

The signals were standardized by setting the signal (c.p.s.) from 5 mM ATP at pH 7.2 as the 100% signal. So y in formula (1) stands for % c.p.s. and x for [ATP]. Figure 5 reveals a pH-dependence of the luciferase light signal of 63% for pH 6.8, 60% for pH 6.7 and 42% for pH 6.3. Under all pH conditions the luciferase signal was saturated at ATP concentrations > 2 mM.

In Figure 6 we demonstrate the estimation of cytoplasmic ATP

Figure 6 Approximate calibration of the luciferase signal from a hepatocyte as cytoplasmic ATP concentration

The cell was superfused with glucose-free Ringer solution to which CN⁻ (4 mM) was added at 0 min. (a) Conversion of the firefly luciferase light signal (after background subtraction) from c.p.s. (left axis) into % of initial c.p.s. (right axis). (b) Calculation of cytoplasmic ATP concentration at pH 7.2 from % c.p.s. by using the calibration curve from Figure 5. (c, d) Expanded plots of 60–70 min showing computed cytoplasmic ATP concentration (μM) if pH were 7.2 (c) or 6.3 (d).

concentrations from the smoothed luciferase light signal from a cell exposed to 4 mM CN⁻ in, as usual, glucose-free Ringer solution. In Figure 6(a) the luciferase signal in c.p.s. (left axis) is shown after subtraction of the photomultiplier background count rate. The initial rise in the signal occurred as superfusion of 45 μM luciferin was started. By averaging this signal over the first 5–30 min of the recording, we have normalized this initial signal as 100 % (right axis of Figure 6a). If intracellular pH were (implausibly) to stay stable at pH 7.2 during the application of CN⁻ we can calculate cytoplasmic [ATP] by solving formula (1) for x ([ATP]), by using the iterated variables a and b from Figure 5:

$$x = \frac{by}{(a-y)} \quad (2)$$

(where y is % c.p.s. from Figure 6a, $a = 113.7$, $b = 0.5$ for pH 7.2). The computed cytoplasmic ATP concentrations are plotted in Figure 6(b). Note that the technique will have poor precision for ATP concentrations above 1 mM (see Figure 5); the spikes in the initial cytoplasmic ATP values almost certainly result from this poor precision and stochastic noise in the signal. Unfortunately, metabolically poisoned cells usually undergo a severe acidosis (Gores et al., 1989); pH 6.2 has been measured in hepatocytes under hypoxic conditions (Herman et al., 1990).

Until the time course of this fall in cytoplasmic pH is known for cells in the particular conditions of our experiments, correction of the luciferase signal for pH changes, and hence more precise estimation of cytoplasmic ATP concentrations, will not be possible. Alternatively it may prove possible to enhance cytoplasmic buffering to abolish intracellular pH changes. At 1 mM ATP or below, the technique will have more acceptable precision. Figures 6(c) and 6(d) show computed cytoplasmic ATP values for this same cell during the plunge in the signal (60–70 min after poisoning), assuming that cytoplasmic pH was 7.2 (Figure 6c) or 6.3 (Figure 6d). Note that cytoplasmic ATP falls to approx. 50 or 100 μM, depending on the chosen pH value. Nevertheless, despite the low cytoplasmic ATP concentration, removal of CN⁻ allowed a recovery of ATP to approx. 2 mM at pH 7.2 (Figure 6b).

The luciferase signal, in conditions that simulate the mammalian intracellular ionic milieu, is saturated at ATP concentrations above about 1–2 mM (Figure 5). The total intracellular ATP content of populations of hepatocytes varies from 2.6 mM (13.2 nmol/10⁶ cells; Harman et al., 1990) to 3.6 mM (17.9 nmol/10⁶ cells; Kane et al., 1985) and 5.6 mM (Charest et al., 1985). Cytoplasmic ATP, as measured by either the digitonin or the non-aqueous fractionation methods (Soboll et al., 1980), constitutes up to 80 % of total ATP in healthy cells. So it is likely that all reported values for cytoplasmic ATP concentrations lie above the concentrations needed to saturate firefly luciferase. This means that changes in the intracellular luciferase signal will only become detectable after a fall in cytoplasmic ATP of between 20 % and 75 %, depending on which of the above measurements are chosen. The inability to monitor cytoplasmic ATP changes above about 1 mM is not particularly crucial for the evaluation of cell damage by inhibition of metabolic energy production, as morphological damage, such as blebbing of the plasmalemma, only occurs at average ATP levels below 10 % of the initial concentration (Harman et al., 1990).

Our data are comparable with some published data on the effects of metabolic inhibition on total ATP in hepatocyte populations; 15 % remaining after 1 h in 0.5 mM CN⁻ (Aw and Jones, 1989), approx. 20 % remaining after 1 h of anoxia (Figure 2 in Gasbarrini et al., 1992a), and 33 % remaining after 1 h anoxia (Gasbarrini et al., 1992b). On the other hand, some published ATP measurements on hepatocyte populations show a much faster decline in total ATP levels; 5 % remaining after 15 min in 1 mM CN⁻ (Sakaida et al., 1992), 15 % remaining after 4 min in 5 mM CN⁻ and 1 mM iodoacetate (Harman et al., 1990). The cell-to-cell heterogeneity in the decline of ATP described here will complicate the interpretation of pathological experiments conducted on cell populations. In the present study, we took precautions to make sure that the microinjection itself did not disturb the cells: we left injected hepatocytes for approx. 30 min in the incubator to recover, and only healthy-looking cells with distinct nuclei and a birefringent, smooth, spherical cell surface as viewed by Normaski optics were then used for experiments.

The data from this new technique show that the decline in cytoplasmic ATP will not occur synchronously in all cells in a population, so the resolution of the role of cytoplasmic ATP in pathophysiological cellular events will require single-cell measurements. By combining luciferase measurements in single cells with simultaneous video-microscopy in i.r. light from a laser diode we have already demonstrated that a sudden shape change in poisoned cardiomyocytes coincides with a sudden loss of luciferase signal (Bowers et al., 1992). We anticipate that, with refined optics, it should be possible to determine whether a critical cytoplasmic ATP concentration coincides with the onset

of plasmalemma blebbing in hepatocytes, and whether restoration of cytoplasmic ATP can reverse the blebbing process.

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REFERENCES

- Anundi, I., King, J., Owen, D. A., Schneider, H., Lemasters, J. J. and Thurman, R. G. (1987) *Am. J. Physiol.* **253**, G390–G396
- Aw, T. Y. and Jones, D. P. (1989) *Am. J. Physiol.* **257**, C435–C441
- Bowers, K. C., Allshire, A. and Cobbold, P. H. (1992) *J. Mol. Cell. Cardiol.* **24**, 211–216
- Charest, R., Blackmore, P. F. and Exton, J. H. (1985) *J. Biol. Chem.* **260**, 15789–15794
- Cobbold, P. H. and Bourne, P. K. (1984) *Nature (London)* **312**, 444–446
- Cobbold, P. H. and Lee, J. A. C. (1991) in *Cellular Calcium: A Practical Approach* (McCormack, J. G. and Cobbold, P. H., eds.), pp. 54–81, Oxford University Press
- DeLuca, M. (1976) *Adv. Enzymol.* **44**, 37–68
- DeLuca, M. and McElroy, W. D. (1974) *Biochemistry* **13**, 921–925
- Elbers, R., Heldt, H. W., Schmucker, P., Soboll, S. and Wiese, H. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 378–393
- Gasbarrini, A., Borle, A. B., Farghali, H., Bender, C., Francavilla, A. and Van Thiel, D. (1992a) *J. Biol. Chem.* **267**, 6654–6663
- Gasbarrini, A., Borle, A. B., Farghali, H., Francavilla, A. and Van Thiel, D. (1992b) *J. Biol. Chem.* **267**, 7545–7552
- Gores, G. J., Nieminen, A.-L., Wray, B. E., Herman, B. and Lemasters, J. J. (1989) *J. Clin. Invest.* **83**, 386–396
- Harman, A. W., Nieminen, A.-L., Lemasters, J. J. and Herman, B. (1990) *Biochem. Biophys. Res. Commun.* **170**, 477–483
- Herman, B., Gores, G. J., Nieminen, A.-L., Kawanishi, T., Harman, A. and Lemasters, J. J. (1990) *CRC Crit. Rev. Toxicol.* **21**, 127–148
- Kane, A. B., Petrovich, D. R., Stern, R. O. and Farber, J. L. (1985) *Am. J. Physiol.* **249**, C256–C266
- Lemasters, J. J., DiGuiseppi, J., Nieminen, A.-L. and Herman, B. (1987) *Nature (London)* **325**, 78–81
- Sakaida, I., Thomas, A. P. and Farber, J. L. (1992) *Am. J. Physiol.* **263**, C684–C690
- Seglen, P. O. (1972) *Exp. Cell. Res.* **74**, 450–454
- Selby, P. L. and Sherratt, H. S. A. (1989) *Trends Pharmacol. Sci.* **10**, 495–500
- Siegmund, B., Koop, A., Kietz, T., Schwartz, P. and Piper, H.-M. (1990) *Am. J. Physiol.* **258**, H285–H291
- Soboll, S., Akerboom, T. P. M., Schwenke, W.-D., Haase, R. and Sies, H. (1980) *Biochem. J.* **192**, 951–954
- Solomonson, L. P. (1981) in *Cyanide in Biology* (Vennesland, B., Conn, E. E., Knowles, C. J., Westley, J. and Wissing, F.), pp. 11–28, Academic Press, London
- Vincent, M. F., Bontemps, F. and Van den Berghe, G. (1992) *Biochem. J.* **281**, 267–272
- Zhang, G. H. and Kraus-Friedmann, N. (1989) *Biochem. Int.* **19**, 333–343

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