Biochem. J. (1989) 263, 347–353 (Printed in Great Britain)

Prolonged high intracellular free calcium concentrations induced by ATP are not immediately cytotoxic in isolated rat hepatocytes

Changes in biochemical parameters implicated in cell toxicity

J. Fred NAGELKERKE,* Peter DOGTEROM, Hans J. G. M. DE BONT and Gerard J. MULDER
Division of Toxicology, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Isolated rat hepatocytes were incubated with ATP to induce high intracellular free Ca^{2+} concentrations as determined with the Quin-2 method. Immediately after addition of ATP, the intracellular concentration of Ca^{2+} rose from 200 nM to more than 2.5 μM. It stayed at this value during the first $\frac{1}{2}$ h; the rise was absolutely dependent on extracellular Ca^{2+}. After the first $\frac{1}{2}$ h the Ca^{2+} concentration decreased to 1–2 μM (5–10 times the control value). These high intracellular free Ca^{2+} concentrations did not lead to an immediate loss of cell viability. Only after 2 h of incubation a substantial number of cells lost viability. This was preceded by a decrease in cellular NADH (> 40%) and accompanied by a sharp increase in the intracellular Ca^{2+} concentration. Under these conditions the NADPH concentration was not affected. Cellular GSH was decreased to 30% of the initial value, but no lipid peroxidation or protein-thiol depletion was observed. Intracellular ATP, ADP and AMP were increased in the presence of extracellular ATP. Ca^{2+}-dependent proteases seemed not to be involved in cell death. These observations are consistent with a collapse of mitochondrial functions as a final trigger of cell death.

INTRODUCTION

The role of intracellular free Ca^{2+} in cytotoxicity induced by xenobiotics has been extensively investigated, especially in hepatocytes [1–5]. Exposure of cells to toxic compounds ultimately leads to elevated free intracellular Ca^{2+} concentrations, followed by cell death. A causal relation between these events has often been suggested. One reason for the increase in cell Ca^{2+} is a toxin-induced disfunctioning of the Ca^{2+} translocators which maintain Ca^{2+} homoeostasis through their capacity to extrude Ca^{2+} from the cell or to sequester it in the mitochondria and endoplasmic reticulum. Thus, at an intracellular free Ca^{2+} concentration of, for instance, 100 μM, various hydrolytic enzymes are activated. This results in changes in the ultrastructure of the cells, swollen mitochondria, condensation of microfilaments and formation of blebs [6]. These concentrations of free Ca^{2+} are, however, reached at a stage when processes leading to cell death have become irreversible, and probably reflect an enhanced permeability of the membrane for extracellular Ca^{2+} [6].

When (hepato)toxins are used to study the consequences of increased Ca^{2+}, they not only interfere with the normal storage or extrusion mechanisms of Ca^{2+} but have usually many other effects on cellular functions and integrity. Krell *et al.* [7] have reported short-term studies in which ATP induced an increase in intracellular Ca^{2+} in hepatocytes through the influx of Ca^{2+}. This increase seems to be mediated through a (Ca^{2+}–Mg^{2+})ATPase present at the cell membrane [8–10]. Orrenius and colleagues have investigated extensively the acute effects of ATP on distribution of intracellular Ca^{2+}; besides an increase in free cytosolic Ca^{2+}, an increase in mitochondrial Ca^{2+} was observed [11]. In other, short-term, experiments the toxicity of ATP and its synergetic effect on toxin-induced cell death were investigated [12–14].

The purpose of this study was to determine the effects of prolonged high Ca^{2+} concentrations on cellular biochemistry; ATP was used to induce the high Ca^{2+} concentration. Hepatocytes were incubated with such concentrations of external ATP that most of the cells remained viable for up to 4 h. Thus it was possible to quantify the intracellular Ca^{2+} concentrations for a prolonged period and study the effects on various biochemical parameters possibly involved in the development of cytotoxicity.

MATERIALS AND METHODS

Materials

Collagenase and ATP were obtained from Boehringer, Mannheim, Germany. Bovine serum albumin (type V), Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)], Quin-2 and Quin-2-AM were obtained from Sigma, St. Louis, MO, U.S.A. Leupeptin was bought from the Peptide Institute, Osaka, Japan. All other reagents were of analytical grade.

Isolation and incubation of hepatocytes

Male Wistar rats of approx. 250 g, from the Sylvius Laboratories, University of Leiden, were used, and had free access to food and water. Liver parenchymal cells were isolated by collagenase perfusion as reported pre-

Abbreviation used: LDH, lactate dehydrogenase.

* To whom correspondence should be sent.

viously [15]. Routinely, over 95% of the freshly isolated hepatocytes excluded Trypan Blue. Cells were incubated at a final concentration of $(2–4) \times 10^6$ cells/ml in Hanks'–Hepes buffer (pH 7.4), supplemented with 1.5% (w/v) bovine serum albumin. Incubations were carried out at 37 °C in a rotary shaker, 200 cycles/min, under O_2/CO_2 (19:1).

All experiments were carried out in triplicate. Typical experiments are presented in the Figures.

Determination of free intracellular Ca^{2+} by Quin-2 fluorimetry

This was done essentially as described in ref. [16]. For the determination of the time course of intracellular free Ca^{2+} during the whole incubation period, it is necessary to have sufficient Quin-2 in the cells. In agreement with other reports [17,18], the indicator was lost from the cells during prolonged incubation: approx. 70% in 1 h. Therefore, to assay free intracellular Ca^{2+} cells were loaded with Quin-2 immediately before determination. At various times a 1 ml sample of the cell suspension was taken from the incubation vessel and transferred to a mini incubation vessel. Then 5 μl of 10 mM-Quin-2-AM in dimethyl sulphoxide was added. After incubation for 10 min at 37 °C, the cells were transferred to a Eppendorf mini test tube and centrifuged at 500 *g* for 5 s; the supernatant was discarded. The cells were resuspended in 3 ml of incubation buffer (final concn. 10^6 cells/ml) and transferred to a cuvette in the Perkin–Elmer LS-5 fluorimeter; excitation wavelength was 339 nm, and emission wavelength 492 nm. The cuvette was kept at 37 °C, and cells were stirred with a mini stirring bar and oxygenated. The fluorescence signal was allowed to stabilize for approx. 1 min before addition of 3 μl of a solution of 5 mM-digitonin to determine maximum fluorescence; minimum fluorescence was recorded after addition of 100 μl of 500 mM-EGTA.

Many compounds interfere with the fluorescence determinations at these excitation and emission wavelengths, either by quenching the light or by autofluorescence. The fluorescence signal in these experiments is in fact composed of autofluorescence of the cells and Quin-2 fluorescence. Therefore, effects of the compounds used on either of these components may influence the final result. However, ATP, dimethyl sulphoxide, EGTA and digitonin had no effect on the autofluorescence of the cells, and ATP and dimethyl sulphoxide did not affect the fluorescence of Quin-2 in buffer. A calibration curve was constructed with Ca^{2+}–EGTA in accordance with ref. [16].

Viability

Cell viability was determined by measuring the leakage of LDH from cells [19] and by determination of the percentage of cells that excluded Trypan Blue [20].

Lipid peroxidation

As an index for lipid peroxidation, the thiobarbituric acid assay was used as described previously [21,22].

Determination of GSH

GSH was determined in sedimented cells (0.5 ml samples) by the colorimetric thiol method of Saville [23].

Determination of protein thiols

Cellular protein thiols were determined with Ellman's reagent as described by Di Monte *et al.* [24]. Samples of the hepatocyte suspension (0.3 ml) were taken and centrifuged for 2 min at 50 *g*. The pellet was washed twice with aq. 6.5% (w/v) trichloroacetic acid and finally suspended in 2 ml of 0.5 M-Tris/HCl buffer, pH 7.6. Ellman's reagent (0.1 ml of a 10 mM solution in methanol) was then added, and after at least 20 min the solution was centrifuged for 3 min at 3000 *g*. The A_{412} of the supernatant was measured.

Determination of NADH and NADPH

This was done essentially as described in refs. [25,26]. Samples of the hepatocyte suspension (1.0 ml) were transferred to pre-cooled (−20 °C) Eppendorf mini test tubes and centrifuged at 500 *g* for 5 s (4 °C). The pellets were immediately frozen in liquid N_2 and kept at −80 °C until work-up.

The pellets were thawed at 4 °C after addition of 200 μl of 0.05 M-KOH containing 5% (v/v) ethanol and 3.5% (w/v) CsCl. After ultrasonication for 5 s, the samples were adjusted to pH 8 with 0.25 M-H_3PO_4 and centrifuged (10000 *g* for 1 min).

NADH was determined by h.p.l.c. A 25 μl sample of the supernatant was injected on a column (15 cm × 0.39 cm) of Nucleosil 120-7 C18 and eluted with a linear gradient (20 min) of 1–5% (v/v) methanol in 0.1 M-potassium phosphate buffer, pH 6.0. General conditions were as follows: flow rate 1.0 ml/min; room temperature; recycling time between runs 10 min; detection at 340 nm. An Apple 2e computer with a Chromatochart program as the controller, two model 510 pumps (Waters Associates) and a model 440 absorbance detector (Waters Associates) were used. All individual samples were assayed in duplicate.

Determination of adenine nucleotides

Samples of the hepatocyte suspension were handled similarly as in the determination of NADH, except that the pellets were thawed after addition of 400 μl of 3 M-$HClO_4$, followed by addition of 100 μl of 1 M-potassium phosphate buffer (pH 8.0) and 210 μl of 10 M-KOH. For determination of ATP, 10 μl of the supernatant was injected on a Nucleosil column (two 15 cm × 0.39 cm columns, coupled in series) with the following program: 10 min at 0% buffer B, then 9 min 10% B, and finally 2 min gradient to 100% B, followed by holding for 9 min at 100% B. Recycle time was 1 min to 0% B and 10 min before the next injection. Buffer A was 0.1 M-potassium phosphate buffer (pH 5.80) and buffer B was 0.1 M-potassium phosphate buffer (pH 5.93) containing 5% methanol. Detection was at 254 nm.

Leupeptin activity

The biological activity of leupeptin was tested as described previously [27]. It showed the expected inhibition activity towards proteases.

Determination of protein content

Protein concentrations were determined as described by Lowry *et al.* [28], with bovine serum albumin as standard.

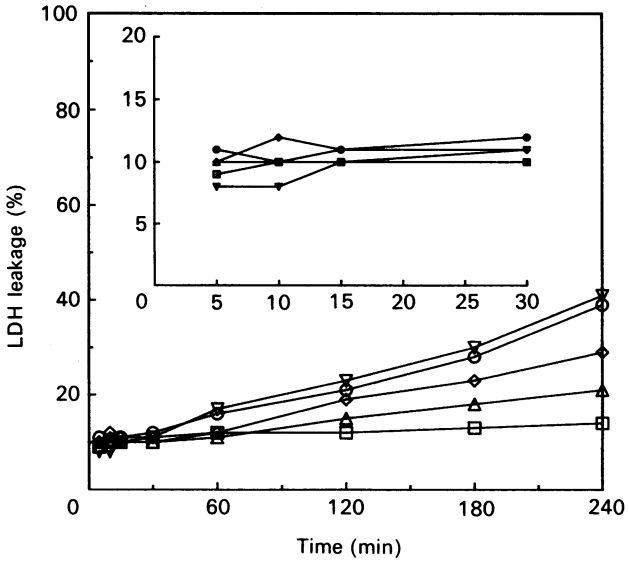

Fig. 1. Effect of extracellular ATP on cell viability as determined by the leakage of the enzyme LDH into the medium

Hepatocytes were incubated with ATP as described in the Materials and methods section. At the indicated time points a 250 μl sample was taken and centrifuged at 50 *g* for 2 min. The LDH activity was determined in the supernatant. Values are expressed as percentages of the total amount present. The total amount was determined by treating the cells from an identical sample with Triton X-100 before centrifugation. □, Control; △, 0.4 mM-ATP; ◇, 0.8 mM-ATP; ○, 1.6 mM-ATP; ▽, 3.2 mM-ATP. Insert shows viability during the first 30 min.

RESULTS

Effect of extracellular ATP on cell viability

Extracellular ATP had relatively little effect on the viability of hepatocytes (Fig. 1); only after more than 2 h loss of LDH occurred, indicating the onset of cell death. Higher concentrations of extracellular ATP increased the leakage.

ADP and AMP also induced cell death. The effect of 1.6 mM-ADP was comparable with that of ATP, but that of 1.6 mM-AMP was less (Fig. 2*a*).

The effect of ATP, ADP and AMP on cell viability was strictly dependent on the presence of extracellular Ca^{2+}: in its absence no loss of viability was observed up to 4 h of incubation (Fig. 2*b*).

The added extracellular ATP was completely hydrolysed in approx. 30 min (Fig. 3); the presence of leupeptin did not affect the rate of hydrolysis (insert in Fig. 3).

It has been reported that the protease inhibitor leupeptin could prevent ATP-induced cell death [11]. However, 100 μg of leupeptin/ml did not affect the rate and extent by which 1.6 mM-ATP induced loss of viability (results not shown).

Effect of ATP on intracellular free Ca^{2+}

As discussed in the Materials and methods section, the leakage of Quin-2 was too fast to allow reliable measurements of Ca^{2+} during the whole incubation period. Therefore, samples were taken at various time points after the start of the incubation, and cells were subsequently loaded with Quin-2 immediately before Ca^{2+} determinations. Only for studies on the immediate effect of ATP was it added directly to Quin-2-loaded cells in the cuvette. Addition of ATP (0.4–3.2 mM) induced an immediate increase in free Ca^{2+} to a value that was above the upper limit of detection by the Quin-2 method (> 2.5 μM). During the next 30 min the concentration decreased to approximately the control value (200 nM) when 0.4 mM- or 0.8 mM-ATP was used. It remained above the upper limit of detection at 3.2 mM. Therefore 1.6 mM-ATP was used to determine the time course of intracellular Ca^{2+} for a longer incubation period. Typical experiments are shown in Fig. 4. The final value reached at 30 min differs from experiment to experiment. The lowest value reached with 1.6 mM-ATP was 1 μM (5 times

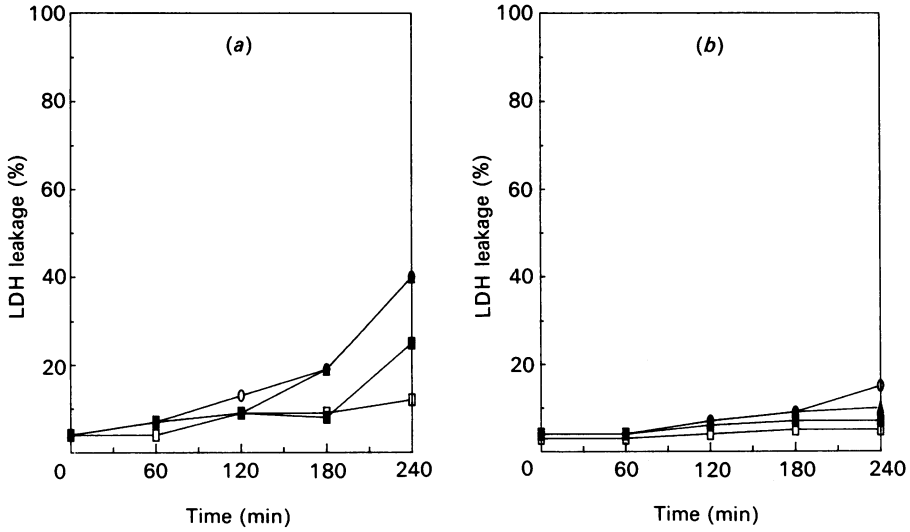

Fig. 2. Effect of extracellular ATP, ADP and AMP on cell viability

(*a*) With extracellular Ca^{2+}; (*b*) without extracellular Ca^{2+}. Determinations were done as described in Fig. 1 legend: □, control; ○, 1.6 mM-ATP; ▲, 1.6 mM-ADP; ■, 1.6 mM-AMP.

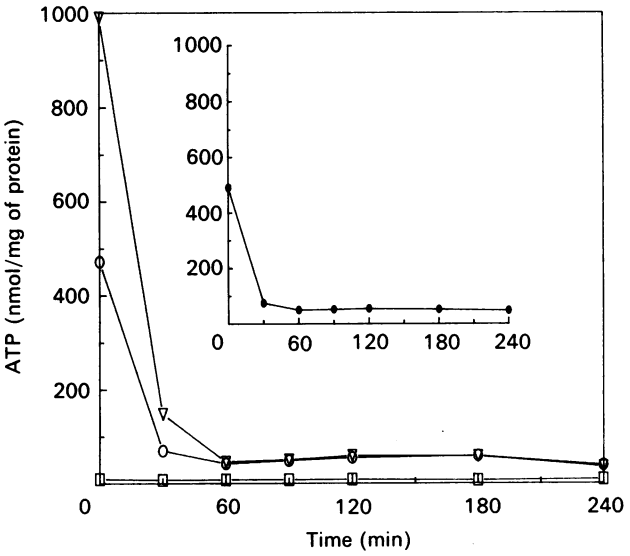

Fig. 3. Hydrolysis of ATP

At the indicated time points a sample was taken from the incubation vessel and immediately frozen. The values shown are therefore the sum of extra- and intra-cellular ATP. ATP was determined by h.p.l.c. Values are expressed per mg of cell protein: □, control; ○, 1.6 mM-ATP; ▽, 3.2 mM-ATP. Insert shows the rate of hydrolysis in the presence of leupeptin and 1.6 mM-ATP.

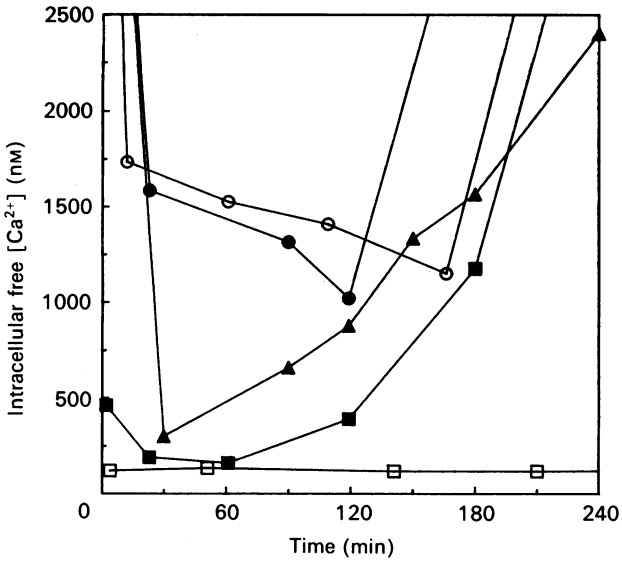

Fig. 4. Effects of adenine nucleotides on intracellular free Ca^{2+} concentrations

Free Ca^{2+} concentrations were calculated from Quin-2 fluorescence values. Determinations were done as described in the Materials and methods section: □, control; ○, 1.6 mM-ATP; ●, 1.6 mM-ATP and 100 μg of leupeptin/ml; ■, 1.6 mM-AMP; ▲, 1.6 mM-ADP.

the control value), but more often it was around 2 μM. Intracellular Ca^{2+} remained at this value for the next 2 h. Then it rose sharply again to values no longer quantifiable by the Quin-2 method. Fig. 4 also shows that leupeptin had no effect on increase in intracellular Ca^{2+} by extracellular ATP. ADP and AMP also induced elevated Ca^{2+} concentrations (Fig. 4). When ADP was added to cells in the cuvette of the fluorimeter the effect was instantaneous, as with ATP. AMP acted more slowly, and the final value reached was not as high as with ATP

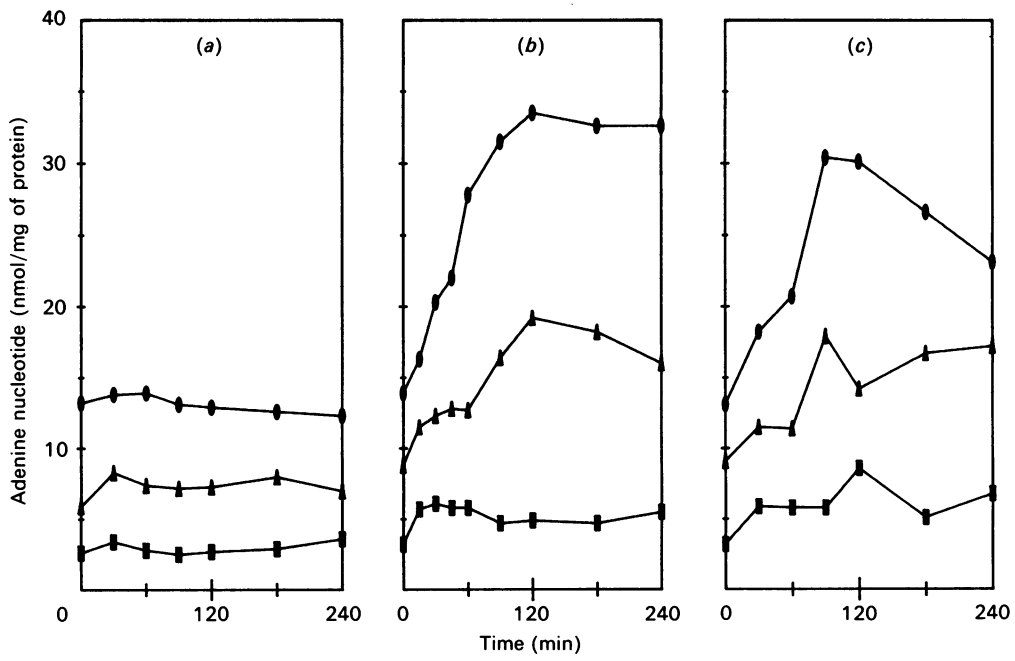

Fig. 5. Effects of ATP on intracellular adenine nucleotides

Samples were taken from the incubation vessel and centrifuged as described in the Materials and methods section. Adenine nucleotides were determined in the pellets by h.p.l.c. after extraction. (*a*) Control; (*b*) exposed to 1.6 mM-ATP; (*c*) exposed to 3.2 mM-ATP. Intracellular: ■, AMP; ▲, ADP; ●, ATP.

and ADP. Eventually, with both ADP and AMP the Ca^{2+} concentration rose to a value above 2.5 μM.

The increase in intracellular Ca^{2+} was strictly dependent on the presence of extracellular Ca^{2+}: in its absence there was no effect of ATP on intracellular Ca^{2+} concentration (results not shown). Also, in the control cells the intracellular concentrations were lower in the absence than in the presence of extracellular Ca^{2+}.

The cells also responded to micromolar concentrations of adenine nucleotides. When 4 μM-ATP was added to Quin-2-loaded cells the Ca^{2+} concentration rose immediately above 2.5 μM; 4 μM-ADP induced an intracellular Ca^{2+} concentration of 1 μM.

Intracellular adenine nucleotide concentrations

Because ATP depletion has been suggested to be one of the causes of cell death, ATP concentrations in cells with sustained high Ca^{2+} concentrations were determined. Before the actual loss of viability (as evidenced by LDH release), we could not find any decrease in ATP in these cells. On the contrary, ATP concentrations tended to be higher in cells with high Ca^{2+} concentrations (Fig. 5). This increase in intracellular ATP was not due to influx of the external added ATP, because addition of 1.6 mM-ADP or -AMP had the same effect (result not shown).

The increase in intracellular ATP was completely dependent on the presence of extracellular Ca^{2+}; in its absence no increase was found. This also proves that the increase in intracellular ATP in the presence of Ca^{2+} is not due to influx of added ATP. Fig. 5 shows that the intracellular ADP and AMP concentrations are also increased in cells exposed to 1.6 mM-ATP (*b*) and 3.2 mM-ATP (*c*).

NADH and NADPH

NADH and NADPH were determined in cells exposed to 1.6 mM- and 3.2 mM-ATP. Especially with the highest concentration, a rapid decrease in NADH occurred (Fig. 6). In contrast, no effects were observed on the NADPH concentrations as a result of exposure to 1.6 mM- or 3.2 mM-ATP.

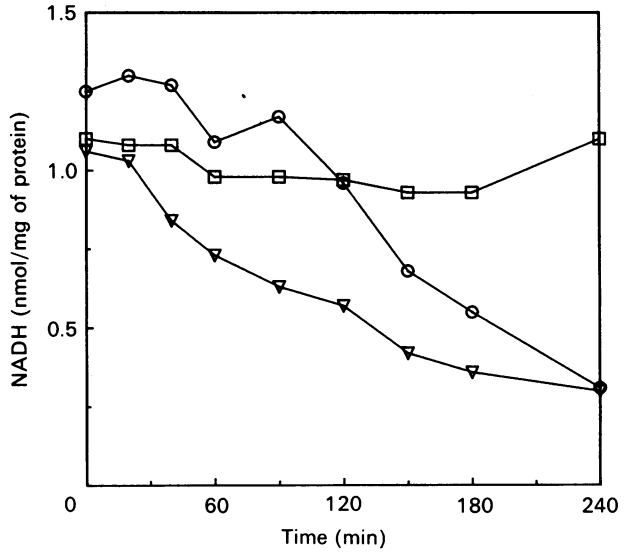

Fig. 6. Effects of ATP on cellular NADH

Samples were taken from the incubation vessel and centrifuged. NADH was determined by h.p.l.c. after extraction from the pellets: □, control; ○, 1.6 mM-ATP; ▽, 3.2 mM-ATP.

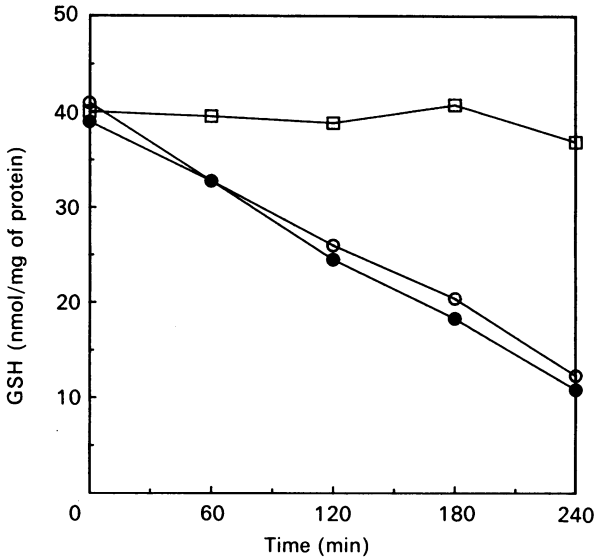

Fig. 7. Effects of ATP on cellular GSH

Samples were taken from the incubation vessel and centrifuged. GSH was determined in the pellets by the colorimetric method of Saville [23]: □, control; ○, 1.6 mM-ATP; ●, 1.6 mM-ATP and 100 μg of leupeptin/ml.

GSH, lipid peroxidation and protein thiols

Prolonged incubation of hepatocytes with 1.6 mM-ATP or 1.6 mM-ATP and 100 μg of leupeptin/ml induced a decrease in intracellular GSH (Fig. 7). No formation of lipid-peroxidation products (malondialdehyde) was observed. Furthermore there was no difference in the content of protein thiols (90–100 nmol/mg of protein) between the control cells and cells exposed to ATP or ATP and leupeptin.

DISCUSSION

This study was undertaken to determine the effects of a sustained high free intracellular Ca^{2+} concentration in hepatocytes. In most studies reported so far, prolonged high Ca^{2+} concentrations were induced by xenobiotics, the metabolism of which resulted in the formation of reactive intermediate that react with essential protein thiol groups. Alternatively, compounds have been used that react directly with protein thiols [5,24,29–31]. However, these compounds act non-specifically, and many biochemical processes may be affected simultaneously under these conditions.

In the present paper ATP was used to induce elevated Ca^{2+} concentrations. Surprisingly, the rate at which cells started to lose viability was relatively slow in spite of the continuous high concentration of free Ca^{2+} inside the cells. Apparently, cells can cope with a 5–10-fold increase in Ca^{2+} over a period of several hours.

We observed a persistent increase in cellular free Ca^{2+} only when extracellular Ca^{2+} was present. Charest *et al.* [9] have reported that stimulation of hepatocytes with

1 μM-ATP also induced a transient increase in intracellular free Ca^{2+} in the absence of extracellular Ca^{2+}, owing to Ca^{2+} mobilization from intracellular pools. We could confirm these results: with 1 μM-ATP, free Ca^{2+} increased to a maximum within a few seconds, remained maximal for about 30 s and then returned to the initial value in 3–4 min. However, the persistent increase in free Ca^{2+} that we observe with 1.6 mM-ATP is completely dependent on extracellular Ca^{2+}. It is therefore most likely to be due to influx from the outside, possibly through the action of a plasma-membrane (Ca^{2+}–Mg^{2+})-ATPase that acts as a Ca^{2+} pump as described by Lin [32].

One of the parameters implicated in cell toxicity is lipid peroxidation, resulting in modification of cellular lipids and/or formation of products that can interact with protein thiol groups [32,33]. In our experiments we observed a slow decrease in GSH, but the concentration remained apparently high enough to prevent lipid peroxidation during the whole incubation period. This excludes lipid peroxidation as the cause of cell death observed at the end of the incubation period. We found no decrease in protein thiols, either.

Another process frequently observed in toxicity is oxidative stress. When this occurs, GSSG is formed, which is subsequently reduced back to GSH at the expense of NADPH. In experiments where oxidative stress was induced in hepatocytes with t-butyl hydroperoxide a dramatic decrease in NADPH was observed [2]. In our experiments the concentration of NADPH did not change, which suggests that the ATP treatment does not lead to oxidative stress, and seems to exclude oxidative stress as the cause of cell death at the end of the incubation period.

The high (millimolar) concentration of ATP used in the present study is not necessary for the increase in the Ca^{2+} concentration: with micromolar concentrations the same value is reached, which is in accordance with Charest *et al.* [9]. However, a high ATP concentration is required to sustain a high Ca^{2+} concentration, since with micromolar concentrations the effect disappears after a few minutes.

Also, ADP and AMP induced increases in cell Ca^{2+}. ADP was as effective as ATP, but the effect of AMP was clearly less. The cause probably is that the effects of ATP and ADP are mediated through a different receptor from those of AMP [9].

It has been suggested that intracellular ATP depletion is the cause of cell death [6]. In the present experiments the high concentrations of Ca^{2+} did not decrease the cellular availability of ATP; the ATP concentration was even higher in the treated cells. At present we have no explanation for this; it is not due to influx of extracellular ATP, because ADP and AMP had the same effect. Furthermore, in the absence of extracellular Ca^{2+} no increase of intracellular ATP was detected. This also seems to exclude an influx of ATP into the cells. Therefore, the observed cell death at the end of the incubation period is not a consequence of a diminished ATP availability.

In cells exposed to ATP the NADH concentration ultimately fell to 30% of that in control cells. A decrease in cellular NADH may have serious consequences for cell viability. Thus studies with isolated mitochondria have demonstrated that Ca^{2+}-loaded mitochondria are very sensitive to oxidation of their NADH [34–36]. When this happens, mitochondrial functional integrity is very rapidly lost, leading to inability to sequester Ca^{2+}. We observed a sharp increase in intracellular Ca^{2+} at the moment that NADH had decreased to 50%. This agrees quite well with the 40% decrease in NADH that was reported to induce Ca^{2+} release from isolated mitochondria [14].

Whereas the added ATP was rapidly removed by hydrolysis, the effect on intracellular Ca^{2+} was permanent; Ca^{2+} was increased instantaneously and remained high during the remainder of the incubation period. Also, the decrease in NADH started within the first ½ h. Yet cell death occurred only after several hours. Probably, as discussed above, NADH has to decrease beyond a critical concentration before cell death occurs.

No effect of the protease inhibitor leupeptin was observed in our experiments, although it has been reported that it provides complete protection against ATP-induced cell death [13]. Our finding is in accordance with a paper by Olafsdottir *et al.* [5], which reported that leupeptin and similarly acting protease inhibitors were unable to prevent Ca^{2+}-induced cytotoxicity. Apparently the cells used by Mirabelli *et al.* [12] and Nicotera *et al.* [13] are much more vulnerable than our cells: although we incubated the hepatocytes with a higher concentration of ATP, it still took 4 h before 50% of the cells had died. Mirabelli *et al.* [12] and Nicotera *et al.* [13] reported that 50% of the cells had died in 30 min.

In conclusion, our results indicate that hepatocytes can survive a major increase in intracellular Ca^{2+} for several hours; however, eventually they die, presumably owing to the loss of mitochondrial functions.

REFERENCES

1. Sies, H., Graf, P. & Estrela, J. M. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3358–3362
2. Bellomo, G., Jewell, S. A., Thor, H. & Orrenius, S. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6842–6846
3. Thor, H., Hartzell, P. & Orrenius, S. (1984) J. Biol. Chem. **259**, 6612–6615
4. Starke, P. E., Hoek, J. B. & Farber, J. L. (1986) J. Biol. Chem. **261**, 3006–3012
5. Olafsdottir, K., Pascoe, G. A. & Reed, D. J. (1988) Arch. Biochem. Biophys. **263**, 226–235
6. Campbell, A. K. (1983) in Intracellular Calcium: its Universal Role as Regulator (Monographs in Molecular Biophysics and Biochemistry) (Gutfreund, H., ed.), pp. 426–429, John Wiley and Sons, Chichester
7. Krell, H., Baur, H. & Pfaff, E. (1979) Eur. J. Biochem. **101**, 349–364
8. Bellomo, G., Mirabelli, F., Richelmi, P. & Orrenius, S. (1983) FEBS Lett. **163**, 136–139
9. Charest, R., Blackmore, P. F. & Exton, J. H. (1985) J. Biol. Chem. **260**, 15789–15794
10. Lin, S. & Russell, W. E. (1988) J. Biol. Chem. **263**, 12253–12258
11. Bellomo, G., Nicotera, P. & Orrenius, S. (1984) Eur. J. Biochem. **144**, 19–23
12. Mirabelli, F., Bellomo, G., Nicotera, P., Moore, M. & Orrenius, S. (1986) J. Biochem. Toxicol. **1**, 29–39
13. Nicotera, P., Hartzell, P., Davis, G. & Orrenius, S. (1986) FEBS Lett. **209**, 139–144
14. Moore, G., Rossi, L., Nicotera, P., Orrenius, S. & O'Brien, P. (1987) Arch. Biochem. Biophys. **259**, 283–295
15. Nagelkerke, J. F., Barto, K. P. & Van Berkel, T. J. C. (1983) J. Biol. Chem. **258**, 12221–12227

16. Rink, T. J. & Pozzan, T. (1985) Cell Calcium **6**, 133–144
17. Berthon, B., Binet, A. & Mauger, J. (1984) FEBS Lett. **167**, 19–24
18. Monck, J. R., Reynolds, E. E., Thomas, A. P. & Williamson, J. R. (1988) J. Biol. Chem. **263**, 4569–4575
19. Seglen, P. O. (1976) Methods Cell Biol. **13**, 29–83
20. Moldéus, P., Hogberg, J. & Orrenius, S. (1978) Methods Enzymol. **51**, 60–71
21. Pascoe, G. A. & Reed, D. J. (1987) Arch. Biochem. Biophys. **256**, 287–296
22. Bergmeyer, H. U., Gawehn, K. & Grassl, M. (1974) in Methods in Enzymatic Analysis (Bergmeyer, H. U., ed.), vol. 1, pp. 481–482, Verlag Chemie, Weinheim
23. Saville, B. (1958) Analyst **83**, 670–672
24. Di Monte, D., Ross, D., Bellomo, G., Eklow, L. & Orrenius, S. (1984) Arch. Biochem. Biophys. **235**, 334–342
25. Schweinsberg, P. D. & Loo, T. L. (1980) J. Chromatogr. **181**, 103–107
26. Jones, D. P. (1981) J. Chromatogr. **225**, 446–449
27. Van Berkel, T. J. C., Kruijt, J. K. & Kempen, H. M. (1985) J. Biol. Chem. **260**, 12203–12207
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
29. Moore, M., Thor, M., Moore, G., Nelson, S., Moldéus, P. & Orrenius, S. (1985) J. Biol. Chem. **260**, 13035–13040
30. Nicotera, P., Hartzell, P., Baldi, C., Bellomo, G. & Orrenius, S. (1986) J. Biol. Chem. **261**, 14628–14635
31. Esterbauer, H. (1985) in Free Radicals in Liver Injury (Poli, G., Cheeseman, K. H., Dianzani, M. U. & Slater, T. F., eds.), pp. 29–47, IRL Press, Oxford
32. Lin, S.-H. (1985) J. Biol. Chem. **260**, 7850–7856
33. Dogterom, P., Mulder, G. J. & Nagelkerke, J. F. (1989) Chem.-Biol. Interact., in the press
34. Lehninger, A. L., Vercesi, A. & Babunmi, E. A. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 1690–1694
35. Mirabelli, F., Salis, A., Marinani, V., Finardi, G., Bellomo, G., Thor, H. & Orrenius, S. (1988) Arch. Biochem. Biophys. **264**, 261–269
36. Lê Quôc, K. & Lê Quôc, D. (1988) Arch. Biochem. Biophys. **265**, 249–257

Received 20 February 1989/30 May 1989; accepted 19 June 1989