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Involvement of intracellular Ca^{2+} and K^{+} in dissipation of the mitochondrial membrane potential and cell death induced by extracellular ATP in hepatocytes

J. Paul ZOETEWEIJ,* Bob VAN DE WATER, Hans J. G. M. DE BONT, Gerard J. MULDER and J. Fred NAGELKERKE
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 extracellular ATP to induce a prolonged increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and a loss of viability within 2 h. By using video-intensified fluorescence microscopy, the effects of exposure to extracellular ATP on $[Ca^{2-}]_i$, mitochondrial membrane potential (MMP) and cell viability were determined simultaneously in individual living hepatocytes. The increase in $[Ca^{2+}]_i$ on exposure to ATP was followed by a decreasing MMP; there were big differences between individual cells. Complete loss of the MMP occurred before cell death was observed. Omission of K^+ from the incubation medium decreased the cytotoxicity of ATP; under these conditions, intracellular K^+ was decreased by more than 80%. Treatment with nigericin also depleted intracellular K^+ and decreased ATP-induced toxicity. Protection against loss of viability by means of a decrease in intracellular $[K^+]$ was reflected by maintenance of the MMP. These observations suggest that ATP-induced cell death may be caused by a mechanism that has been described for isolated mitochondria: after an increase in Ca^{2+} levels, a K^+ influx into mitochondria is induced, which finally disrupts the MMP and leads to cell death.

INTRODUCTION

The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) is involved in the regulation of several cellular processes such as cell division, cell differentiation, metabolism and secretion [1–3]. In hepatocytes, $[Ca^{2+}]_i$ is kept at a resting value of approx. 200 nM, which is 10000-fold less than the Ca^{2+} concentration outside the cell, in the blood. To maintain the $[Ca^{2+}]_i$ at this level, Ca^{2+} is extruded from the cells by Ca^{2+}/Mg^{2+}-ATPases located in the plasma membrane. Ca^{2+} can also be sequestered into cellular compartments, i.e. mitochondria and the endoplasmic reticulum [3,4]. Uptake of Ca^{2+} into mitochondria is driven by the negative membrane potential over the mitochondrial inner membrane. Release of Ca^{2+} from liver mitochondria into the cytosol occurs mainly by Ca^{2+}/H^+ exchange.

A rise in $[Ca^{2+}]_i$ can be induced in several ways. For instance, physiological increases are induced by vasoactive peptides and α-adrenergic agonists, which bind to receptors on the plasma membrane, resulting in stimulation of Ca^{2+} influx into the cells and mobilization of Ca^{2+} from the endoplasmic reticulum [5–8]. Non-physiological increases can be induced by certain xenobiotics, e.g. vanadate [9], which inhibits the plasma-membrane Ca^{2+}/Mg^{2+}-ATPase.

Exposure of isolated hepatocytes to extracellular 0.4 mM-ATP induces a prolonged highly increased $[Ca^{2+}]_i$, accompanied by an increase in intracellular ATP, ADP and AMP concentrations. Prevention of the influx of Ca^{2+}, by omission of Ca^{2+} from the extracellular medium, abolishes the cytotoxicity caused by extracellular ATP [10].

Much work has been done during recent years to investigate the role of elevated $[Ca^{2+}]_i$ in cell injury [11–13]. A reason for these studies was the finding that cells contained high levels of Ca^{2+} after exposure to many toxic compounds [14,15]. One hypothesis was that increased $[Ca^{2+}]_i$ in hepatocytes activated Ca^{2+}-dependent degradative enzymes in the cytosol, e.g. proteases, the activity of which rapidly led to cell death [16]. However, cell death due to exposure of hepatocytes to ATP, which induces a substantial increase in $[Ca^{2+}]_i$, could not be prevented by protease inhibitors [10,17]. More recently it has been suggested that extensive Ca^{2+} cycling [18] over the mitochondrial inner membrane, or a Ca^{2+}-induced permeability transition of the inner membrane [19–21], will lead to loss of mitochondrial function and, ultimately, cell death.

Isolated mitochondria accumulate Ca^{2+} when an excess of Ca^{2+} is offered. As a result, a selective K^+ channel [22] or a non-specific pore, which is sensitive to cyclosporin A [1,22,23], is opened in the mitochondrial membrane. The influx of K^+, driven by the mitochondrial membrane potential (MMP), induces swelling of the mitochondria. A similar sustained influx of K^+ can also be achieved with the K^+ ionophore valinomycin. The K^+ influx will finally cause depolarization of the mitochondrial membrane [24].

In this paper, a large increase in $[Ca^{2+}]_i$ is induced by exposure to 0.4 mM-ATP, and effects on mitochondrial function are investigated by studying the relation between $[Ca^{2+}]_i$, MMP and cell viability simultaneously in individual living hepatocytes. Furthermore, in this study we have investigated whether the Ca^{2+}-dependent effects measured with isolated mitochondria may be involved in the mechanism of cytotoxicity of extracellular ATP in intact hepatocytes.

MATERIALS AND METHODS

Materials

Collagenase, BCECF-AM and ATP were obtained from Boehringer, Mannheim, Germany. BSA (fraction V), poly-D-

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; pH_i, intracellular pH; LDH, lactate dehydrogenase; MMP, mitochondrial membrane potential; VIFM, video-intensified fluorescence microscopy; BCECF, 2′,7′-bis-(2-carboxyethyl)-5 (and 6)-carboxyfluorescein penta-acetoxymethyl ester.

* To whom correspondence should be addressed.

lysine hydrobromide (M > 452000), rhodamine 123, propidium iodide, nigericin, ionomycin, Fura-2 and Fura-2-AM were obtained from Sigma, St. Louis, MO, U.S.A.

Isolation and incubation of hepatocytes

For all experiments, male Wistar rats (approx. 200 g) from the Sylvius Laboratories, University of Leiden, were used. The rats had free access to food and water. Liver parenchymal cells were isolated by collagenase perfusion as reported previously [25]. Viability of freshly isolated cells was > 95%, as measured by exclusion of Trypan Blue [26]. For cell-suspension experiments, the cells were incubated at a final concentration of 3×10^5 cells/ml in Hanks'/Hepes buffer (pH 7.4), which is composed of 120 mM-NaCl, 5 mM-KCl, 4.2 mM-$NaHCO_3$, 1.2 mM-NaH_2PO_4, 2.6 mM-$CaCl_2$, 0.5 mM-$MgSO_4$ and 25 mM-Hepes, supplemented with 1% (w/v) glucose and 1.5% (w/v) BSA. Incubation of cell suspensions was carried out at 37 °C in a rotary shaker, at 200 cycles/min, under O_2/CO_2 (19:1). All experiments were done in triplicate unless otherwise indicated.

Lactate dehydrogenase (LDH) determination

Cell viability in cell-suspension experiments was determined by measuring the leakage of LDH from the cells. A 1 ml portion of the cell suspension was taken and centrifuged for 10 s at 600 ***g***. The amount of LDH in the supernatant was assayed spectrophotometrically as described previously [27]. The total amount of LDH present in the cells was measured after treatment of the cells with a drop of 20% (v/v) Triton X-100 in water.

Attachment of hepatocytes

For microscopic experiments, cells were attached to circular glass coverslips, which fit into the incubation chamber of the microscope. Before use, the glass coverslips were coated with poly-D-lysine by the following procedure: the coverslips were washed with a mixture of ethanol/diethyl ether (1:1, v/v) and dried. Then they were covered with 50 μl of a 1 mg/ml solution of poly-D-lysine in water for 20 min; finally the coverslips were washed with water and dehydrated with a series of ethanol/water mixtures of 75%, 90% and 100% (v/v) ethanol sequentially.

For attachment, 1.5 ml of a cell suspension (1.5×10^5–3×10^5 cells/ml in Hanks'/Hepes buffer) was pipetted into each well of a six-well culture plate in which a coated glass coverslip was placed at the bottom. The plates were put in an incubator at 37 °C under an atmosphere of air/CO_2 (19:1) for 30 min. Then the coverslips were washed three times with Hanks'/Hepes buffer. Approx. 5×10^5 cells had attached per coverslip.

Video-intensified fluorescence microscopy (VIFM)

Fluorescence measurements in single freshly isolated hepatocytes attached to poly-D-lysine-coated glass coverslips were performed by using a VIFM system. The system consists of a Zeiss IM-35 inverted microscope (Oberkochen, Germany) equipped with a Nikon Fluor 40× oil objective. Excitation source was a 50 W mercury arc lamp. The exposure time was determined by a computer-controlled shutter. A heat filter and neutral-density filters were placed in the excitation light path. The excitation filters were mounted in a computer-controlled spinning filter wheel or a moving filter block which also contained the mirrors and emission filters. Images were collected with a CCD series 200 camera system (Photometrics, Tucson, AZ, U.S.A.) controlled by a Compaq 386/20 computer (Compaq Computer Corporation, Houston, TX, U.S.A.). The digitized images were processed and analysed on an Imagine system (Synoptics, Cambridge, U.K.). Digitized pictures were stored on an Optistar optical disk (Corel Systems Corp., Ottawa, Canada).

For $[Ca^{2+}]_i$ measurements, hepatocytes were loaded during the attachment period with 10–20 μM-Fura-2-AM. For determination of the mitochondrial membrane potential, the cells were co-loaded with 500 nM-rhodamine-123. After 30 min, during which the cells were attached and loaded with the fluorescent probes, the cells were washed three times with Hanks'/Hepes buffer. Then the glass coverslip was mounted in an incubation chamber on the microscope. The cells were kept at 37 °C under an atmosphere of O_2/CO_2 (19:1).

Experiments were performed in Hanks'/hepes buffer containing 150 nM-propidium iodide, which stains the nuclei of non-viable cells. Cells were preincubated for 15 min before addition of ATP. Background fluorescence was measured at a site where no cells were attached. Autofluorescence was measured in non-loaded cells (< 10%). Exposure time to excitation light was as short as possible to minimalize photobleaching.

For intracellular Ca^{2+} measurements, the cells were excited at a Ca^{2+} insensitive and a Ca^{2+}-sensitive wavelength of 340 nm and 380 nm respectively, combined with a 395 nm dichroic mirror and 470 nm long-pass filter. All images were corrected for background and autofluorescence. Subsequently the 340 nm image was divided by the 380 nm image on a pixel-by-pixel basis. The mean values of pixel ratios for individual cells were determined in order to calculate the intracellular Ca^{2+} concentration by using the equation of Grynkiewicz *et al.* [28]; for the construction of a standard curve, pixel ratios were determined by using the same conditions and equipment with Fura-2-containing Ca^{2+}/EGTA buffers.

The MMP was measured by using a 450–490 nm excitation filter, a 510 nm dichroic mirror and a 520 nm long-pass filter. The relative fluorescence was calculated by addition of all pixel values of a cell and dividing this total by the sum of the pixel values measured at $t = 0$.

Cells were inspected for propidium iodide fluorescence in the nuclei by using a 510–560 nm excitation filter, a 580 nm dichroic mirror and a 590 nm long-pass filter.

Fluorescence of the different probes did not interfere with each other under the conditions described. Every experiment was performed in triplicate, and five to ten cells were analysed per experiment.

Intracellular K^+ determination

Samples (10 ml) were taken from the cell suspensions and centrifuged for 1 min at 600 ***g***. The supernatant was removed, and 1 ml of 6.5% (w/v) trichloroacetic acid was added to the remaining cell pellets. The samples were centrifuged for 10 min and the intracellular K^+ concentration was measured in the supernatant with a Corning clinical flame photometer 410C (Halstead, Essex, U.K.). Contamination by extracellular K^+ was negligible.

The cell pellets contained both living and dead cells. Dead cells contain almost no K^+. To calculate the K^+ concentration in living cells, the K^+ concentration determined was corrected for the fraction of cells that were dead. This fraction was determined by LDH leakage at the time of sampling for the K^+ assay. The correction was made by dividing the K^+ concentrations by the fraction of cells that were living.

Intracellular pH (pH_i) determination

Samples (3 ml) were taken and incubated with 1 μM-BCECF-AM for 10 min. Then the cells were centrifuged for 1 min at 600 ***g*** and resuspended with incubation medium containing 0.2% BSA in a 3 ml cuvette and stirred at 37 °C. Fluorescence was

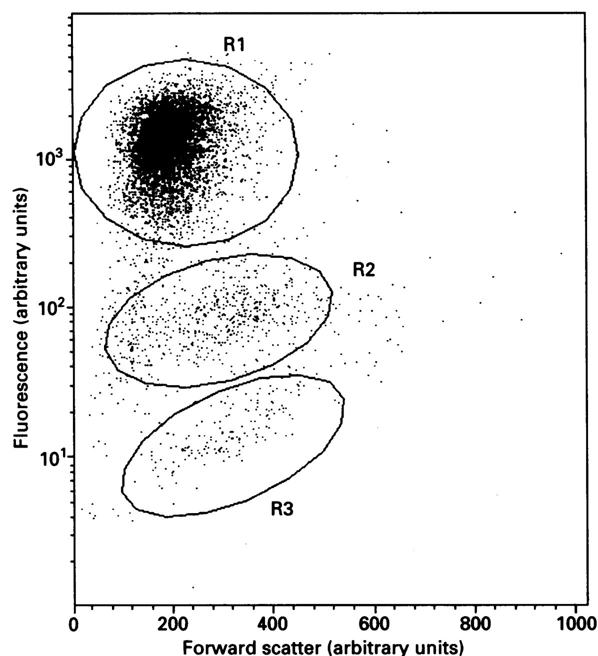

Fig. 1. Flow-cytometric analysis of hepatocytes loaded with 1 μM-rhodamine-123

Control hepatocytes (> 95% viability) were loaded with 1 μM-rhodamine-123. The fluorescence of each cell (arbitrary units), reflecting MMP, is plotted against its forward scatter (arbitrary units), which is related to size and shape. The fluorescence is given on a logarithmic scale. R1–R3, regions 1–3.

measured at 440 nm and 490 nm with a Perkin–Elmer LS-5B luminescense spectrometer. The 440 nm/490 nm ratio was used to determine the pH_i. To calculate the pH_i values from the measured ratios, a standard curve was constructed *in situ* by loading hepatocytes with BCECF-AM and measuring the ratio in cells with a modified incubation buffer containing 10 μM-nigericin, KCl instead of NaCl and external pH ranging from 6.7 to 8.0 [29].

Flow-cytometric analysis of the MMP

For determination of the MMP in flow-cytometric studies, 0.5 ml of the cell suspensions were loaded with 5 μl of an aq. 100 μM-rhodamine-123 solution for 10 min and directly analysed on the flow cytometer. Cells were analysed for their fluorescence and scattering on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.) with a focused argon laser. Autofluorescence was determined without loading the cells and was approx. 1%.

The flow rate was set at 12 μl/min. Each analysis was performed on 5×10^3 particles. With the use of a data-management system (LYSYS software; Becton Dickinson), data were analysed and stored. The fluorescence of each particle was plotted on a logarithmic scale in arbitrary units against its forward scatter also in arbitrary units (dotplot).

Three populations could be distinguished, with respect to the fluorescence intensity (Fig. 1). Manually, regions R1–R3 were defined which contained more than 98% of the cells. The number of cells within the defined regions and their individual fluorescence were determined. After a standard loading of non-treated hepatocytes with rhodamine-123, a highly fluorescent population corresponding to approx. 90% of the cells fell in region 1 (R1), a second small population (< 10%) which exhibited over 10-fold lower fluorescence, representing cells with no MMP, fell in region 2 (R2), and a third small population (< 5%) with hardly any fluorescence, representing cell and tissue fragments, was in region 3 (R3).

When non-viable cells were measured, more than 90% of the cells accumulated in region 2.

The percentage of the cells accumulating in the first population divided by the total number of cells detected was used to calculate the number of cells which maintained an MMP. The average fluorescence of the first population after treatment was also compared with their initial fluorescence.

RESULTS

Effect of exposure to ATP on intracellular Ca^{2+} and MMP

The effect of extracellular ATP on $[Ca^{2+}]_i$ and MMP was examined in individual living hepatocytes by VIFM. Cells were

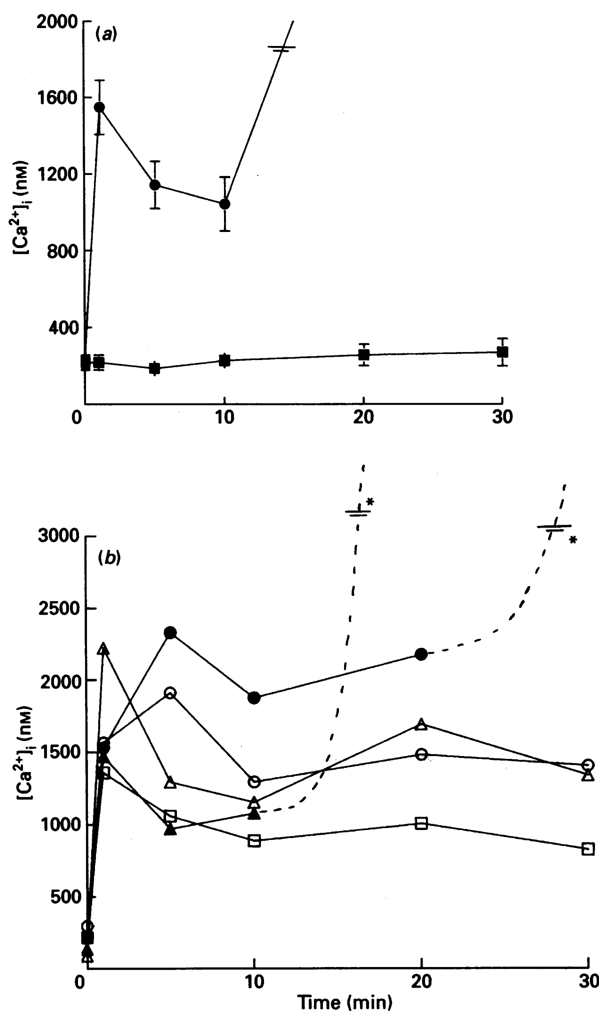

Fig. 2. Effect of ATP on $[Ca^{2+}]_i$ in individual hepatocytes

(*a*) Isolated cells were attached to poly-lysine glass coverslips and mounted in the microscope. The cells were loaded with Fura-2-AM to measure $[Ca^{2+}]_i$. At various time points images were taken and the fluorescence intensities were measured. ■, Control; ●, 0.4 mM-ATP. Data are representative of one of three separate experiments and are means ± S.E.M. of five to ten cells. (*b*) Five cells from the experiment in (*a*) are individually shown. Open symbols represent cells that survived during the experiment. Closed symbols represent cells that died as indicated by uptake of propidium iodide. *Indicates excessive Fura dye leakage from the cell and an increase in fluorescence above the quantification level.

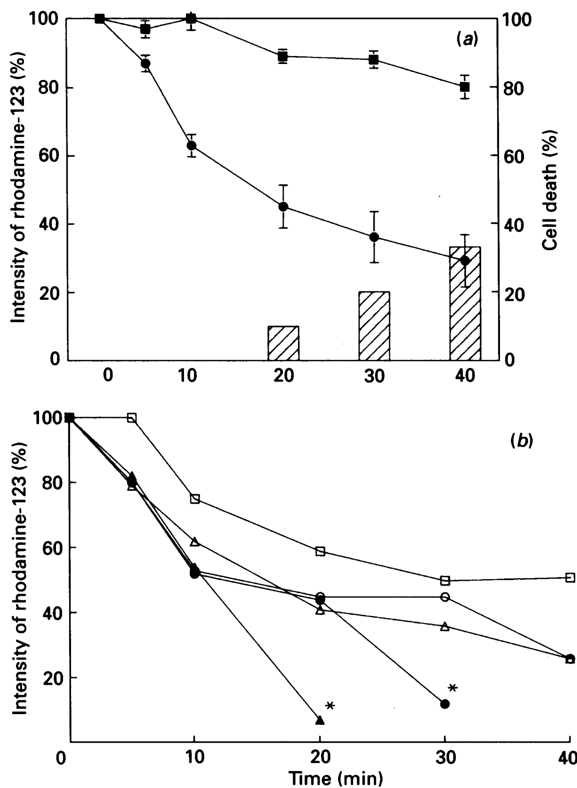

Fig. 3. Effect of ATP on MMP and viability in individual hepatocytes

(*a*) Data obtained in the same cells as in Fig. 2. The cells were loaded with rhodamine-123 for measurement of MMP. Propidium iodide was added to the incubation medium. Left axis: MMP as percentage accumulation of rhodamine-123; ■, control; ●, 0.4 mM-ATP. Right axis: percentage of the cells that exhibited propidium iodide fluorescence in the nucleus; ▨, 0.4 mM-ATP. The experiment was performed until 35% of the cells lost their viability. No loss of viability occurred in the control. (*b*) The MMP of the same five individual cells as shown in Fig. 2(*b*). *Uptake of propidium iodide in the nucleus was observed.

loaded with the fluorescence probes, attached to coated glass coverslips and incubated so that they could be observed in the microscope. Fluorescence images were collected that reflect $[Ca^{2+}]_i$, MMP and cell viability. After exposure to 0.4 mM-ATP, the $[Ca^{2+}]_i$ increased instantaneously to above 1000 nM (Fig. 2*a*) as reported previously for cell suspensions [10]. During the subsequent 10 min, the $[Ca^{2+}]_i$ decreased, although it remained far above the basal level. Then in some cells it increased further to levels well above the detection limit, causing a large increase in the average $[Ca^{2+}]_i$. In other cells, this rapid rise did not occur during the time of the experiment (Fig. 2*b*). These results indicate that averaging of $[Ca^{2+}]_i$ over the total cell population (as has been done in Fig. 2*a*) is meaningless, because clearly there were now two different populations of cells. Exposure to ATP substantially decreased the MMP, and uptake of propidium iodide (indicating cell death) in an increasing number of cells became evident (Fig. 3*a*).

The cells that showed the secondary rapid rise in $[Ca^{2+}]_i$ were those that also lost their MMP completely (Figs. 2*b* and 3*b*). Individual cells that survived exposure to ATP during the experiment retained a measurable MMP, although they maintained a highly elevated $[Ca^{2+}]_i$.

The onset of cell death was preceded by the second sharp increase in $[Ca^{2+}]_i$ and leakage of Fura dye into the medium, both due to permeabilization of the plasma membrane [13]. During these events, the MMP fell to virtually zero (Fig. 3*b*), after which uptake of propidium iodide occurred.

Flow-cytometrical analysis was also used to study the effect of ATP on the MMP in individual cells in cell suspensions. Cells were loaded for 10 min and 1 μM-rhodamine-123 and directly analysed on the flow cytometer. The number of cells that completely lost MMP after 2 h of exposure to 0.4 mM-ATP (Table 1, part A) correlated well with the number that lost viability as measured by the amount of LDH that had leaked out of the cells (results not shown). The average MMP of the remaining still-viable cells was significantly decreased compared with control values.

Table 1. Effect of omission of extracellular K^+ on ATP-induced dissipation of MMP

Isolated hepatocytes were incubated in Hanks'/Hepes buffer with or without K^+ ($[K^+]_o = 0$), and exposed to 0.4 mM-ATP. Flow-cytometric analysis of 0.5 ml samples loaded with 1 μM-rhodamine-123 was used to calculate the percentage of cells that maintained an MMP (percentage of cells accumulating in population R1; see Fig. 1). The average MMP ($MMP_{av.}$) of these cells (population R1) was calculated by averaging their fluorescence, and presented as the percentage of the control at $t = 0$. Data shown are mean values ± S.E.M. of three separate isolations.

	A $[K^+]_o = 5$ mM		B $[K^+]_o = 0$ mM	
	−ATP	+ATP	−ATP	+ATP
Percentage of cells with MMP				
$t = 0$	92±1		91±1	
$t = 120$ min	89±1	34*±4	89±1	61*†±5
$MMP_{av.}$ (% of control at $t = 0$)				
$t = 0$	100		105±2	
$t = 120$ min	90±4	64*±6	99±6	87±8

* $P < 0.05$ compared with control without ATP.
† $P < 0.05$ compared with current value in the presence of K^+.

Role of intracellular K^+

Two methods were used to decrease $[K^+]_i$ to investigate the involvement of intracellular K^+ in the ATP-induced effects. Firstly, K^+ was left out of the incubation medium. Samples were taken for measurement of $[K^+]_i$, LDH leakage and flow-cytometric analysis of the MMP. The initial $[K^+]_i$ was decreased by 40% when the cells were preincubated for 30 min in Hanks'/Hepes buffer without K^+ (Fig. 4*a*). Exposure to ATP in the absence of extracellular K^+ resulted in an additional loss of K^+, which decreased the $[K^+]_i$ after 60 min to 15% of the control values. When the normal 5 mM-K^+ was present in the incubation medium, ATP did not alter the $[K^+]_i$, which is probably due to a compensatory reuptake of K^+ from the medium. When K^+ was left out of the incubation medium, a significant decrease in ATP cytotoxicity was observed after 120 min (Fig. 4*b*). Under these conditions, a significant increase in the amount of cells maintaining their MMP on exposure to ATP was found (Table 1, part B). Also the average MMP of the cells which maintained an MMP was only slightly decreased compared with control values.

In order to test whether a sustained increase in $[Ca^{2+}]_i$ induced by a different mechanism also resulted in K^+-dependent cytotoxicity, cells were exposed to the Ca^{2+} ionophore ionomycin. An almost instantaneous sustained increase in $[Ca^{2+}]_i$, similar to that obtained with ATP, was also achieved with 20 μM-ionomycin

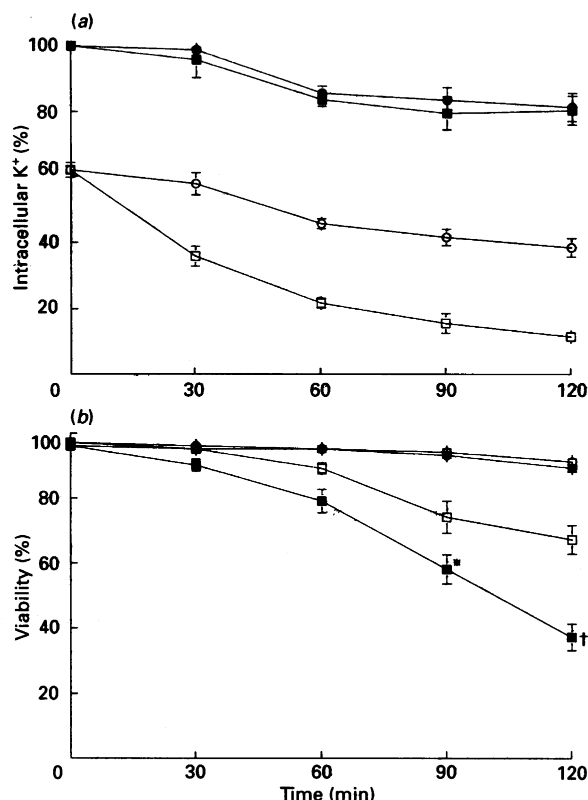

Fig. 4. Effect of omission of extracellular K+ on the intracellular K+ concentration (a) and ATP-induced cell death (b)

(a) At various time points, $[K^+]_i$ was determined. Data are corrected for the percentage of cells that lost viability, as measured by LDH leakage. Control (●) and 0.4 mM-ATP (■) in the presence of extracellular K+; control (○) and 0.4 mM-ATP (□) in the absence of extracellular K+. (b) Viability of cells of various time points. Control (●) and 0.4 mM-ATP (■) in the presence of extracellular K+; control (○) and 0.4 mM-ATP (□) in the absence of extracellular K+. Data are means±S.E.M. of five separate isolations. *$P < 0.05$, †$P < 0.005$, compared with exposure to ATP in the absence of extracellular K+ (two-tailed Student's t test).

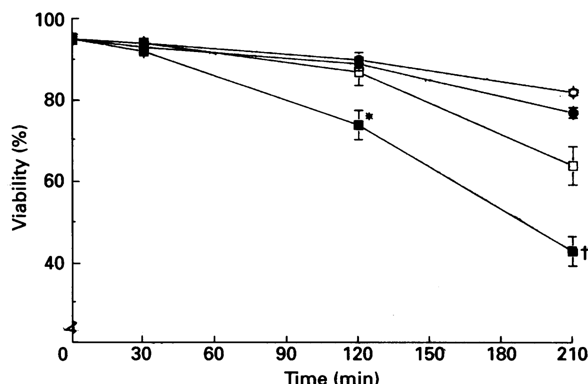

Fig. 5. Effect of omission of extracellular K+ on cell death induced by ionomycin

Control (●) and 20 μM-ionomycin (■) in the presence of extracellular K+; control (○) and 20 μM-ionomycin (□) in the absence of extracellular K+. Data given are means±S.E.M. of three to four separate isolations. *$P < 0.05$, †$P < 0.02$, compared with exposure to ionomycin in the absence of extracellular K+.

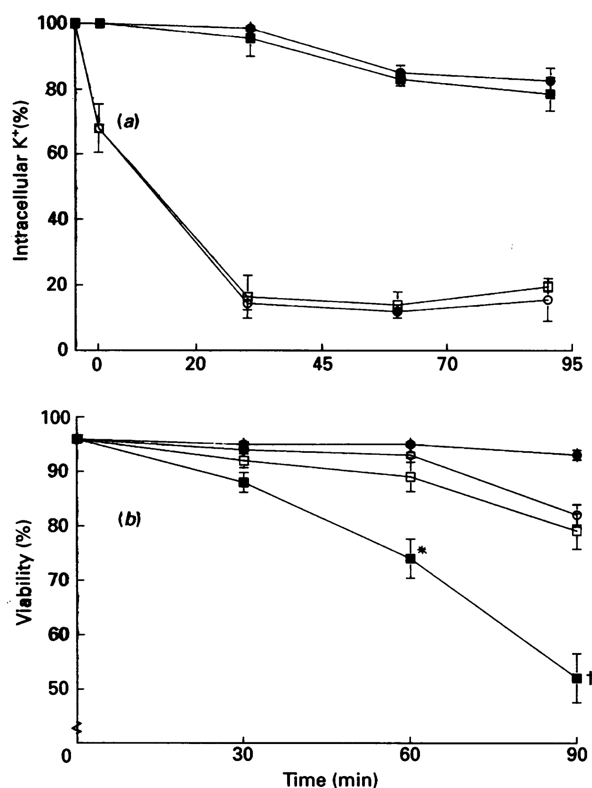

Fig. 6. Effect of nigericin on intracellular K+ concentration (a) and ATP-induced cell death (b)

(a) ●, control; ○, 10 μM-nigericin; ■, 0.4 mM-ATP; □, 0.4 mM-ATP+nigericin. Nigericin was added 5 min before $t = 0$. Data are corrected for loss of viability. (b) ●, control; ○, 10 μM-nigericin; ■, 0.4 mM-ATP; □, 0.4 mM-ATP+nigericin. Data given represent means±S.E.M. of five separate isolations. *$P < 0.01$, †$P < 0.001$, compared with exposure to ATP+nigericin.

Table 2. Effect of increased pH_o on the intracellular pH

Isolated hepatocytes were incubated (see the Materials and methods section) in Hanks'/Hepes buffer at two different pH_o values and the pH_i was measured after treatment. Data shown are mean values±S.E.M. of three separate isolations.

Treatment	Intracellular pH	
	$pH_o = 7.4$	$pH_o = 7.7$
Control	7.36±0.03	7.58±0.02
Nigericin (10 μM)	7.13±0.02	7.48±0.04

(results not shown). Cell death caused by 20 μM-ionomycin was significantly decreased when K+ was omitted from the incubation medium (Fig. 5).

In a second approach, $[K^+]_i$ was decreased with the use of nigericin, a K^+/H^+ antiporter. Addition of 10 μM-nigericin caused a more than 80 % depletion of $[K^+]_i$ within 30 min (Fig. 6a). The length of the experiment was limited to 90 min because of cytotoxicity induced by nigericin itself (Fig. 6b). However, cell death caused by ATP was clearly decreased by nigericin.

Role of cytosolic acidification

It has been demonstrated that an acidic pH_i can protect against cytotoxicity [30]. Because nigericin exchanges K+ for H+, cytosolic acidification might play a role in the protection observed against ATP-induced toxicity. Therefore the pH_i was measured

Table 3. Effect of nigericin on ATP-induced cell death at different pH_o values

Isolated hepatocytes were incubated (see the Materials and methods section) in Hanks'/Hepes buffer at two different pH values. Viability was measured after treatment using the LDH assay. Data shown are mean values ± S.E.M. of three to five separate isolations.

Treatment	Viability at $t = 0$ min (%)		Viability at $t = 90$ min (%)	
	pH 7.4	pH 7.7	pH 7.4	pH 7.7
Control	97 ± 0.3	97 ± 0.3	94 ± 1	94 ± 1
Nigericin (10 μM)			82 ± 2	80 ± 3
0.4 mM-ATP			58 ± 4	55 ± 4
0.4 mM-ATP + nigericin			78* ± 2	71* ± 4

* $P < 0.05$, compared with exposure to 0.4 mM-ATP without nigericin.

with BCECF-AM after addition of nigericin. Treatment with 10 μM-nigericin indeed decreased the pH_i (Table 2).

To examine the possibility that the protective effect of nigericin was due to this cytosolic acidification, pH_i was increased by changing the extracellular pH (pH_o) [31]. An increase in pH_i was observed in both control and nigericin-treated cells when the pH_o was adjusted to 7.7 (Table 2). The increase in pH_i did not change cytotoxicity induced by 0.4 mM-ATP, and protection by nigericin at high pH_i remained essentially the same (Table 3). This suggests that cytosolic acidification has no or only a minor role.

DISCUSSION

Previously we have shown that extracellular ATP induces a concentration-dependent prolonged rise in $[Ca^{2+}]_i$, and ultimately causes cell death [10]. ATP binds to purinergic receptors on the plasma membrane, resulting in the opening of Ca^{2+} channels through which Ca^{2+} enters the cell [32]. During the sustained increase in $[Ca^{2+}]_i$, the concentrations of all adenine nucleotides in the cell (including ATP) also greatly increased. Cell death did not occur when the influx of Ca^{2+} was prevented by omission of Ca^{2+} from the extracellular medium [10]. It was suggested that a loss of mitochondrial function induced by high $[Ca^{2+}]_i$ rather than elevation of $[Ca^{2+}]_i$ itself caused cell death; the NADH concentration was greatly decreased. Therefore in the present paper the effects of extracellular ATP on $[Ca^{2+}]_i$, MMP and cell viability were further examined.

A large increase in $[Ca^{2+}]_i$ (> 1000 nM) caused by exposure to 0.4 mM-ATP was followed by a decreasing MMP and a progressive loss of viability. Loss of viability, as detected by means of propidium iodide uptake, occurred after the MMP was totally dissipated. The mechanism by which the greatly increased $[Ca^{2+}]_i$ plays a role in ATP-induced cell death is still unclear. Ca^{2+}-induced excessive influx of K^+, the major intracellular cation, into mitochondria can be suggested as a possible mechanism. Loading of isolated mitochondria with a large amount of Ca^{2+} enhanced the permeability of the inner membrane specifically for K^+ [22] or non-specifically by pore opening induced by membrane protein ADP-ribosylation [1]. Ca^{2+}-dependent activation of mitochondrial phospholipase A_2 has also been implicated in changes in the permeability of the inner membrane [20].

In order to study the role of intracellular K^+ in ATP-induced cytotoxicity in hepatocytes, we manipulated the $[K^+]_i$ by omission of extracellular K^+ and by addition of nigericin. A decreased $[K^+]_i$ (which did not influence the ATP-induced Ca^{2+} influx into the cell; results not shown) resulted in a significant decrease in the cytotoxicity caused by ATP. Cell death caused by ionomycin, another agent that induced a lasting increase in $[Ca^{2+}]_i$, was also dependent on $[K^+]_i$.

More protection was provided by nigericin than by omission of extracellular K^+, which may be explained by the fact that in the latter case the $[K^+]_i$ is more slowly decreased. It is also possible that nigericin acts as a K^+/H^+ antiporter at the mitochondrial level, preventing accumulation of K^+ in the mitochondria. There is no or only a minor contribution of cytosolic acidification to the protective effect of nigericin. Decreasing the $[K^+]_i$ prevented the loss of MMP similarly to the way in which it prevented cell death. This is in agreement with the observations (VIFM) in individual cells that exposure to ATP decreases the MMP and that a total loss of MMP occurs just before the cell dies.

In summary, the data in the present paper suggest that high $[Ca^{2+}]_i$ induced by ATP in hepatocytes is associated with loss of MMP as well as cell death. In isolated mitochondria, Ca^{2+} overload induces membrane damage, allowing entry of K^+. Such a mechanism may explain the dependence on $[K^+]_i$ of the observed mitochondrial damage and loss of viability induced by extracellular ATP.

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