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Level of Cytosolic Free Calcium during Acetaminophen Toxicity in Mouse Hepatocytes

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SUMMARY

It has been suggested that elevated cytosolic free calcium plays a key role in acetaminophen-induced cell death. The present study has examined the effect of a toxic concentration of acetaminophen on cytosolic free calcium in single mouse hepatocytes, using the dye fura-2 and video imaging fluorescence microscopy. Cytosolic free calcium was calculated from the ratio of emitted fluorescence at >475 nm produced by excitation at 340 and 380 nm, using a double-intensified silicon target camera and digital image processing. In the presence of 5 mM acetaminophen, cell death did not occur for 2 hr, but the toxic lesion that ultimately killed the cells occurred as early as 1 hr. If cytosolic free calcium plays an important role in these toxic events, it would be expected to increase during this period. However, during a 2-hr exposure, cytosolic free calcium concentration in cells exposed to acetaminophen was not different from control. In hepatocytes incubated for longer than 2 hr, the calcium concentration increased shortly before loss of cell viability (i.e., as a late event), consistent with an influx of calcium through a damaged cell membrane. This late increase in calcium occurred well after the appearance of cell surface blebs. The data suggest that there is no sustained change in cytosolic free calcium in acetaminophen injury either before or during the time when irreversible toxic events occur in hepatocytes.

The toxic effects of acetaminophen are believed to be due to the formation of an electrophilic metabolite, NAPQI (1). The nature of the hepatotoxicity initiated by this metabolite has been the subject of much debate in recent years (2, 3). One proposal (4) has been that acetaminophen toxicity involves a disturbance of the processes involved in controlling $[Ca^{2+}]_i$.

The resting $[Ca^{2+}]_i$ in hepatocytes is about 100–200 nM, and its regulation appears to be important for the maintenance of a number of cell functions (5). Transient increases in $[Ca^{2+}]_i$ of the order of 200–400 nM have been shown to be associated with the control of a number of cellular processes (6). Interference with the ability of the cell to control these Ca^{2+}-associated functions could affect cell viability adversely. It has been proposed that acetaminophen toxicity involves inhibition of enzymes that help maintain the large calcium concentration gradient across the cell membrane. This could result from the interaction of NAPQI with critical thiol groups on these enzymes (4), with their subsequent inability to buffer $[Ca^{2+}]_i$ then resulting in an uncontrolled increase in concentration. It has been proposed that this increase may then lead to deleterious effects, such as activation of Ca^{2+}-dependent degradative enzymes (phospholipases, proteases, and endonucleases) and/or Ca^{2+}-dependent changes to the cytoskeleton (2).

There are two main arguments supporting this hypothesis. Firstly, acetaminophen toxicity *in vivo* has been correlated with a decrease in plasma membrane Ca^{2+}-ATPase activity (7, 8) and a decrease in ATP-dependent Ca^{2+} accumulation in plasma membrane vesicles isolated from acetaminophen-poisoned rats (9). Secondly, exposure to high concentrations of acetaminophen results in an increase in phosphorylase *a* activity both *in vivo* (10, 11) and in isolated hepatocytes (4). This enzyme can be activated by increased $[Ca^{2+}]_i$ via phosphorylase *b* kinase, which in turn converts phosphorylase *b* to phosphorylase *a* (12). Hence, the increase in phosphorylase *a* activity has been commonly used as an indirect indicator of a rise in $[Ca^{2+}]_i$. However, there have been no measurements of intracellular calcium itself in hepatocytes to support these ideas.

Recent developments in low-light video fluorescence microscopy have allowed a more direct estimation of calcium in single cells, using fluorescent probes (13). In the present study, we have measured $[Ca^{2+}]_i$ in single mouse hepatocytes, using the calcium-indicating dye fura-2 together with digitized video microscopy, in order to determine whether the early events associated with the development of acetaminophen toxicity involve an alteration in $[Ca^{2+}]_i$.

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ABBREVIATIONS: NAPQI, *N*-acetyl-*p*-benzoquinoneimine; $[Ca^2]_i$, cytosolic free calcium concentration; KRH, Krebs-Ringer-HEPES buffer; LDH, lactate dehydrogenase; ANOVA, analysis of variance; HEPES, 4-(2-hydroxyethyl)-1-piperazine-*N*-(2-ethanesulfonic) acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Materials and Methods

Hepatocyte preparation. Hepatocytes were isolated from male Swiss mice (30–35 g) by collagenase digestion of the liver, as described previously (14). They were suspended in RPMI 1640 medium containing 10 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin, pH 7.4, at a concentration of 12 mg of wet weight/ml, and were plated onto 25-mm-diameter collagen-coated tissue culture dishes. Cells to be used for fluorescence microscopy were added to dishes containing a collagen-coated glass coverslip. After a 2-hr incubation in a 5% CO_2 atmosphere at 37°, the cells were rinsed with 3 × 2 ml of phosphate-buffered saline and 2 ml of KRH containing (in mM) 115 NaCl, 5 KCl, 1 KH_2PO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, and 25 Na-HEPES buffer, pH 7.4. Acetaminophen was dissolved directly in the buffer before incubation with the cells. Subsequent incubations were carried out at 37° in an air atmosphere, in the absence or presence of acetaminophen (1, 5, or 10 mM), in KRH.

Measurement of $[Ca^{2+}]_i$ in single cells. Fluorescence measurements in single hepatocytes were performed using digitized video microscopy, on an Olympus IMT-2 inverted fluorescence microscope modified with quartz optics. Excitation light was provided by a 100-W mercury vapor lamp and passed through either a 340- or 380-nm narrow band-pass interference filter selectable by computer control. Excitation intensity was attenuated by 94–97% using neutral density filters. Fluorescent images were directed via a low-light, intensified silicon-intensified target video camera (model 66; Dage-MTI, Michigan City, IN) to a computer (IBM 386), where they were digitized with an FG-100 video acquisition and display board (Imaging Technologies, Woburn, MA) for background subtraction, ratioing, and storage.

For $[Ca^{2+}]_i$ measurements, hepatocytes were incubated with 2 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min and then rinsed three times with 2 ml of KRH, and the cells attached to the coverslip were transferred to an incubation chamber (Metaltec, Raleigh, NC) on the heated stage (37°) of the microscope. Fluorescence was imaged through a 400-nm dichroic reflector and 475-nm long-pass filter. Images were obtained in adjacent empty areas of the coverslip to correct for background noise. After background subtraction, a ratio of intensities was obtained by dividing the image at 340 nm by the image at 380 nm, on a pixel by pixel basis. The $[Ca^{2+}]_i$ was determined from the following equation:

$$[Ca^{2+}]_i = K_d \cdot (R - R_{min})/(R_{max} - R) \cdot (S_a/S_b)$$

as given by Grynkiewicz (15), where K_d = 224 nM, R = ratio of 340/380-nm fluorescence, R_{min} = 340/380-nm fluorescence ratio in zero Ca^{2+}, R_{max} = 340/380-nm fluorescence ratio in the presence of saturating Ca^{2+}, S_a = fluorescence intensity at 380 nm in zero Ca^{2+}, and S_b = fluorescence intensity at 380 nm in saturating Ca^{2+}. R_{min} plus S_a and R_{max} plus S_b were determined in hepatocytes incubated in the presence of Ca^{2+}-free (KRH containing 3 mM EGTA but no added calcium) or 1 mM Ca^{2+} buffers, respectively, in the presence of ionomycin (10 μM) or Br-A23187 (1 μM) to permeabilize the cell membrane to Ca^{2+}. Autofluorescence (fluorescence from nonloaded cells) was negligible (<1%).

Using this method, $[Ca^{2+}]_i$ could be either determined in a number of cells in the field, to yield an average concentration at one time point, or measured in a single cell over an extended incubation period, to follow temporal changes in $[Ca^{2+}]_i$. In some experiments, hepatocytes were exposed to 5 mM acetaminophen for 2 hr and loaded with dye 30 min before measurement of $[Ca^{2+}]_i$ (i.e., after 90 min). These were then washed to remove the bathing medium and further incubated either in KRH or in Ca^{2+}-free KRH (containing 3 mM EGTA) on the microscope stage, and $[Ca^{2+}]_i$ was monitored in a single hepatocyte, in the presence of acetaminophen, until loss of viability. Under these conditions, the sudden loss of fura-2 fluorescence from the cell was used as an indicator of the loss of membrane integrity.

Assays. Phosphorylase *a* activity in the cells on the tissue culture dishes was determined as described previously (16, 17). An index of cell viability was determine by expressing LDH activity in the bathing medium as a percentage of total activity after cell lysis by sonication (14). Protein was determined by the method of Hartree (18).

Results

Measurement of acetaminophen toxicity and phosphorylase *a* activity in hepatocytes. Fig. 1 details the cytotoxic effects of acetaminophen (1–10 mM) on isolated mouse hepatocytes over a 4-hr incubation in KRH. Between 0 and 2 hr there was no loss of cell viability (Tukey's test, $p > 0.05$), but cell death increased markedly after this period. Whereas the loss of cell viability did not occur until after 2 hr, phosphorylase *a* activity increased much earlier (Fig. 2). Phosphorylase *a* activity produced by exposure to 5 and 10 mM acetaminophen was greater than control within 30 min (ANOVA, $p < 0.05$) and continued to increase with further incubation.

Because acetaminophen toxicity is associated with inhibition

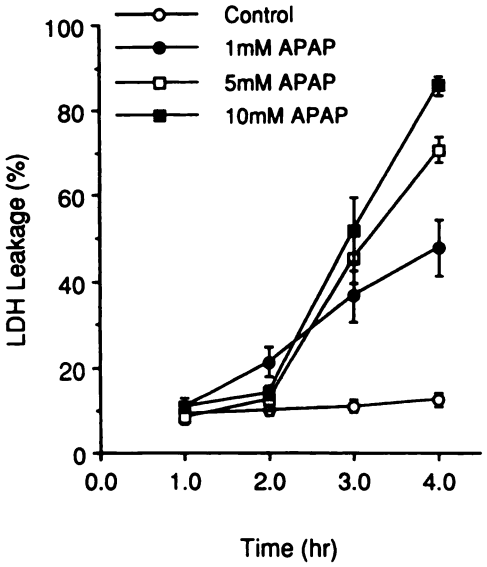

Fig. 1. Effect of acetaminophen (*APAP*) on the time course of LDH leakage from mouse hepatocytes over a 4-hr incubation period. Values are mean ± standard error of five experiments.

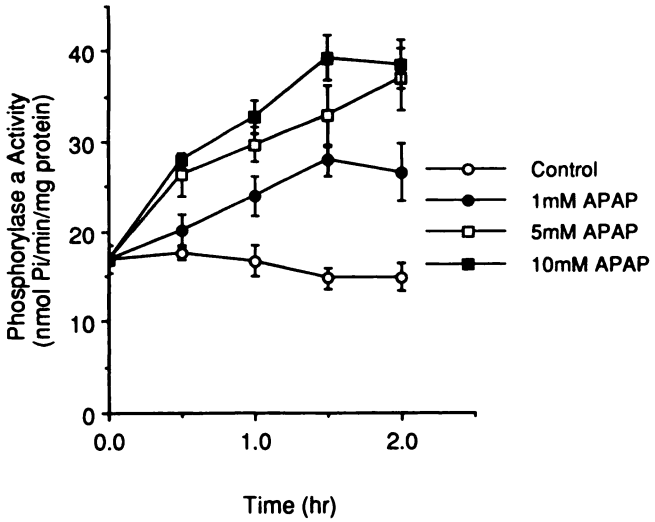

Fig. 2. Effect of acetaminophen (*APAP*) on the time course of phosphorylase *a* activity in mouse hepatocytes over a 2-hr incubation period. Values are mean ± standard error of five experiments.

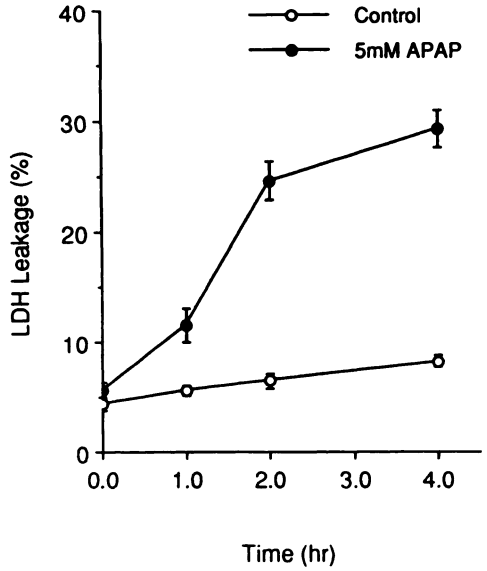

Fig. 3. Leakage of LDH from mouse hepatocytes pre-exposed to 5 mM acetaminophen (*APAP*). Hepatocytes were exposed to acetaminophen for 1 hr (phase 1) and washed, and incubation was continued in acetaminophen-free buffer (phase 2). The data show the LDH leakage during phase 2. Values are mean ± standard error of five experiments.

TABLE 1

Effect of acetaminophen on LDH leakage and phosphorylase *a* activity in hepatocytes in the presence and absence of fura-2

Hepatocytes were incubated in KRH in the absence or presence of 2 μM fura-2 acetoxymethyl ester for 30 min, washed, and subsequently incubated in the absence (controls) or presence of 5 mM acetaminophen for up to 4 hr. Values are mean ± standard error (four experiments).

	LDH leakage		Phosphorylase *a*, 1.5 hr[a]
	2 hr	4 hr	
	%		*nmol/min/mg*
Control	8 ± 1	10 ± 1	13 ± 1
Control + fura-2	2 ± 1	2 ± 1	15 ± 1
Acetaminophen	9 ± 1	68 ± 3	45 ± 3
Acetaminophen + fura-2	3 ± 1	69 ± 2	47 ± 4

[a] nmol of phosphate formed/min/mg of cell protein.

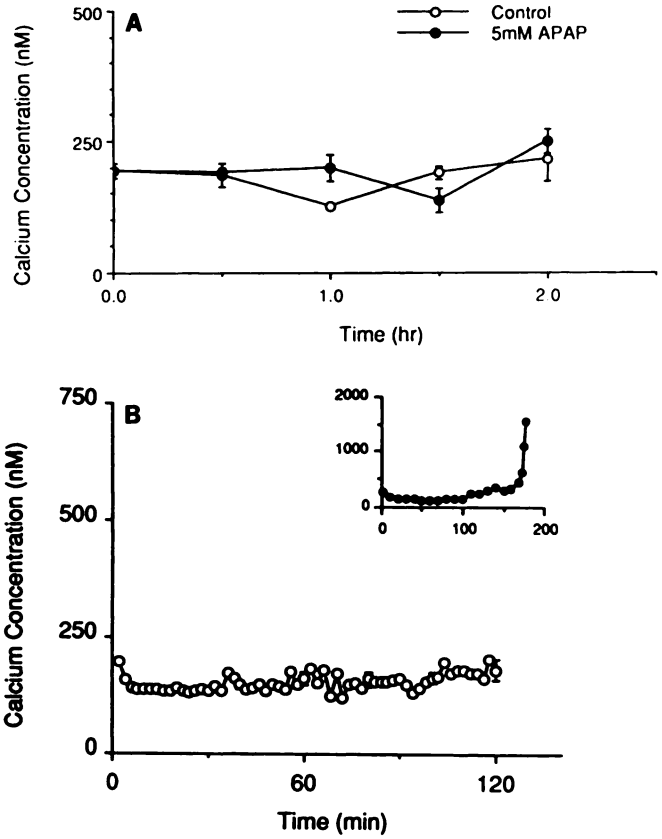

Fig. 4. A, Effect of exposure time in the presence and absence of 5 mM acetaminophen (*APAP*) on $[Ca^{2+}]_i$ in single hepatocytes. Hepatocytes were loaded with fura-2 30 min before measurement of calcium. Values are mean ± standard error of at least 20 individual cells per estimation from at least four different cell preparations. B, $[Ca^{2+}]_i$ in single mouse hepatocytes exposed to 5 mM acetaminophen. Values are mean of four individual cells in which measurements of calcium were recorded every 2 min over a 2-hr period. For clarity, the standard error is shown on every fifth point. There was no significant effect of incubation time on $[Ca^{2+}]_i$ ($F = 1.4$; degrees of freedom = 1180; $p > 0.05$). Likewise, $[Ca^{2+}]_i$ did not change in control cells over this period (data not shown). *Inset*, time course of $[Ca^{2+}]_i$ in an individual cell from the beginning of exposure to acetaminophen until loss of membrane viability at 180 min.

of oxidative phosphorylation (19, 20), the effect of other mitochondrial inhibitors on phosphorylase *a* activity was examined. After a 90-min incubation in KRH, cyanide (2 mM), antimycin A (4 μM), and rotenone (5 μM) each increased phosphorylase *a* activity in mouse hepatocytes, from 24 ± 3 nmol of phosphate formed/min/mg of cell protein in controls (mean ± standard error) to 37 ± 2, 45 ± 1, and 38 ± 1, respectively (three experiments).

If intracellular Ca^{2+} plays a crucial role in the development of acetaminophen toxicity, then changes in its concentration should occur early in the poisoning process. The data in Fig. 3 demonstrate that the toxic events resulting in loss of cell viability can take place as early as 1 hr after exposure to acetaminophen. Hepatocytes were exposed to 5 mM acetaminophen for 1 hr (phase 1) and washed (3 × 2 ml), and incubation was continued in acetaminophen-free medium (phase 2). The washing procedure removed essentially all the acetaminophen from the cells (21). Fig. 3 shows the LDH leakage from hepatocytes during the latter phase. Although there was no loss of viability during phase 1 in the presence of acetaminophen (see Fig. 1), >25% of the cells died within the next 4 hr of incubation in acetaminophen-free KRH. These data confirm previous studies (21–24), which have demonstrated that a brief (approximately 1 hour) exposure to acetaminophen is sufficient to injure cells irreversibly at a later time, and indicate that the damaging events that occur during phase 1 are sufficiently toxic to result ultimately in cell death. If calcium plays a central role in these processes, it would be expected that changes in $[Ca^{2+}]_i$ would occur within the first hour of incubation.

Measurement of $[Ca^{2+}]_i$ in single hepatocytes. Incubation of mouse hepatocytes in 2 μM fura-2 acetoxymethyl ester for 30 min resulted in diffuse fluorescent labeling of the cell. The acetoxymethyl ester is cleaved by intracellular esterases to yield the cell-impermanent fura-2 salt (14). Treatment of hepatocytes with 20 μM digitonin, which selectively permeabilizes the cell membrane (25), released >99% of cellular fluorescence. This indicated that the fura-2 was localized largely in the cytosolic compartment. $[Ca^{2+}]_i$ in individual hepatocytes under control conditions ranged from 104 to 335 nM, with a mean ± standard error of 191 ± 10 ($n = 44$). This value is similar to those reported by others in isolated rat hepatocytes (5, 26). One concern was that the presence of fura-2 in these cells may alter the toxic response to acetaminophen. However, LDH

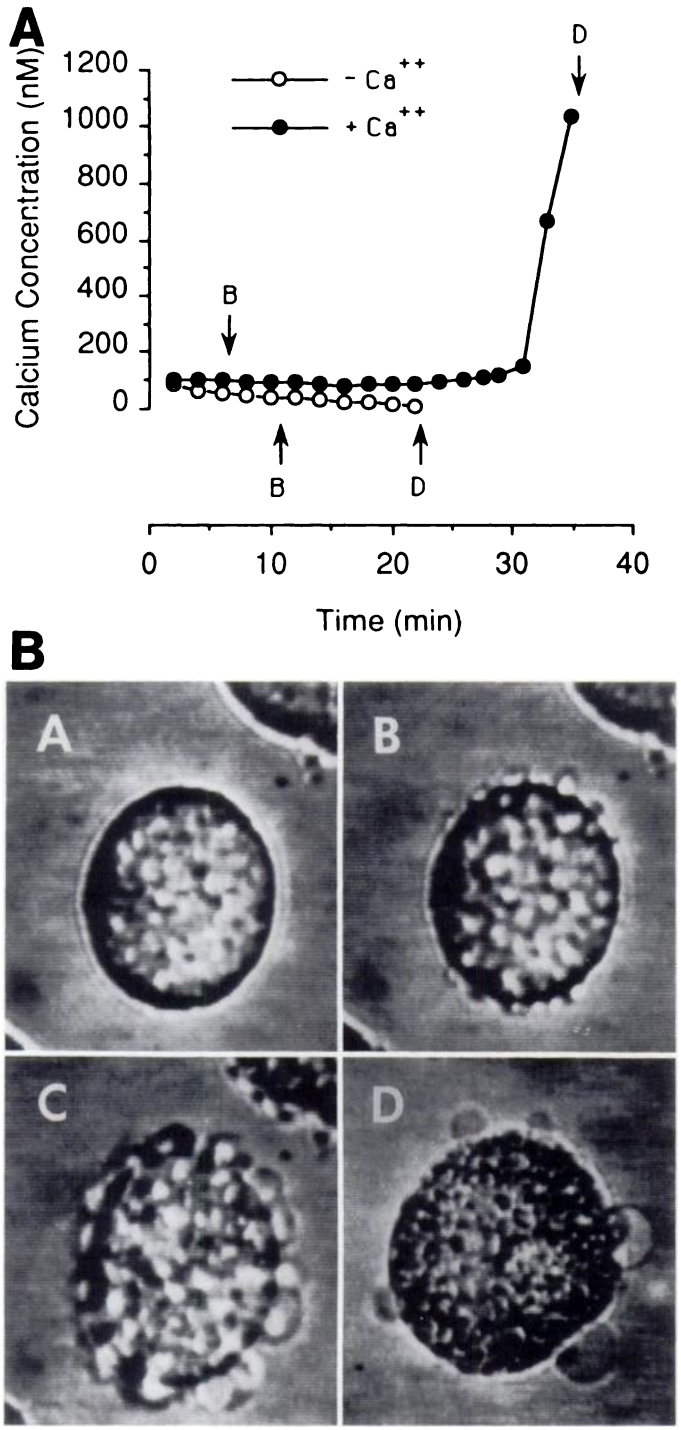

Fig. 5. A, $[Ca^{2+}]_i$ in single mouse hepatocytes after exposure to acetaminophen. Hepatocytes attached to a collagen-coated coverslip were exposed to 5 mM acetaminophen for 2 hr in KRH. Fura-2 loading was commenced 30 min before the calcium measurements shown. The coverslip was then transferred to the incubation chamber of the microscope stage, and incubation was continued either in KRH (●) or in calcium-free KRH (○), both of which contained 5 mM acetaminophen. *B*, The first appearance of cell surface blebs; *D*, loss of cellular fluorescence. The results represent data from individual cells and are typical of at least five experiments, although the time until loss of fluorescence varied considerably from cell to cell. In contrast, control cells (not exposed to acetaminophen at any stage) did not exhibit abrupt elevations in $[Ca^{2+}]_i$. B, Bleb formation during the late stages of acetaminophen toxicity. The two hepatocytes studied in A were imaged by phase microscopy between measurements of $[Ca^{2+}]_i$. After a 2-hr exposure to acetaminophen, the cell surface was unblebbed (A) and $[Ca^{2+}]_i$ was 105 nM. In calcium-containing KRH, cell surface blebs appeared after 6 min (B) but $[Ca^{2+}]_i$ was unchanged (110 nM). After 30 min, $[Ca^{2+}]_i$ increased abruptly to >1 μM and at this time there was extensive blebbing (C). After 35 min the cell lost fluorescence, indicating loss of membrane patency. D, Hepatocyte morphology after 14-min incubation in calcium-free KRH. In this cell there was evidence of extensive cell surface blebbing due to acetaminophen exposure, without an increase in $[Ca^{2+}]_i$.

leakage and the increase in phosphorylase *a* produced by 5 mM acetaminophen were the same in fura-2-loaded cells and cells not loaded with the dye (Table 1).

In order to determine whether $[Ca^{2+}]_i$ changes early in the poisoning process, $[Ca^{2+}]_i$ was measured in hepatocytes during the first 2 hr of incubation with 5 mM acetaminophen. In marked contrast to the increase in phosphorylase *a* activity, the data in Fig. 4A demonstrate that $[Ca^{2+}]_i$ was no different from control values during this period (ANOVA, $p > 0.05$). In addition, $[Ca^{2+}]_i$ was monitored in individual hepatocytes at 2-min intervals (Fig. 4B). There was no evidence to indicate that either a transient or sustained increase in $[Ca^{2+}]_i$ occurs during the first 2 hr of exposure to acetaminophen. Hence, in these cells, increased phosphorylase *a* activity was not an indicator of increased $[Ca^{2+}]_i$.

Agonists such as epinephrine, phenylephrine, and vasopressin produce characteristic increases in $[Ca^{2+}]_i$ in hepatocytes (5, 26). It was found that, after a 2-hr incubation in KRH, mouse hepatocytes were capable of responding to agonist-induced increases in $[Ca^{2+}]_i$. Addition of epinephrine (10 μM) to the bathing medium increased $[Ca^{2+}]_i$ from 268 ± 63 nM to 779 ± 97 nM (three experiments) within 60 sec. Acetaminophen exposure did not alter this agonist-induced increase. After a 2-hr exposure to 5 mM acetaminophen, epinephrine increased $[Ca^{2+}]_i$ from 257 ± 20 to 647 ± 118 nM (three experiments). Hence, after a 2-hr exposure to acetaminophen, these hepatocytes were still capable of responding to agonist-linked increases in $[Ca^{2+}]_i$. Furthermore, these data indicate that it is unlikely that the failure to observe an increase in $[Ca^{2+}]_i$ after acetaminophen exposure was due to an alteration in the calcium-dependent fluorescence properties of fura-2.

In order to determine whether changes in $[Ca^{2+}]_i$ play a significant role late in the poisoning process, $[Ca^{2+}]_i$ was measured in single hepatocytes from the 2-hr exposure time point until the cells lost fluorescence (taken to be loss of cell membrane viability). Data in Fig. 5A illustrate the time-course of $[Ca^{2+}]_i$ in representative single hepatocytes after a 2-hr exposure to 5 mM acetaminophen. In calcium-containing KRH buffer, $[Ca^{2+}]_i$ was essentially unchanged until shortly before loss of membrane viability. At this time, there was an abrupt increase in $[Ca^{2+}]_i$ to >1 μM. Within a few minutes there was a rapid loss of fluorescence, indicating diffusion of dye out of the cell through a leaky cell membrane. In a calcium-free KRH buffer, there was no increase in $[Ca^{2+}]_i$ before loss of cell membrane viability. This indicated that the source of the increase in $[Ca^{2+}]_i$ in the calcium-containing buffer was an influx of extracellular calcium through the damaged cell membrane, rather than calcium released from the intracellular stores.

The formation of cell surface blebs caused by agents that induce oxidative stress has been attributed to an effect of calcium on the cell cytoskeleton (27). In the present study, bleb formation did not correlate with this rise in $[Ca^{2+}]_i$ (Fig. 5). In

calcium-containing buffer, bleb formation preceded the increase in $[Ca^{2+}]_i$. In addition, bleb formation also occurred in cells incubated in calcium-free KRH, where blebbing occurred shortly before loss of membrane viability in the absence of a change in $[Ca^{2+}]_i$.

Discussion

Two important findings with respect to acetaminophen toxicity in mouse liver cells have arisen from the present study. Firstly, during a time when acetaminophen produces an irreversible toxic lesion in the cell, there is no sustained change in $[Ca^{2+}]_i$. Secondly, the data indicate that phosphorylase *a* activity does not necessarily reflect $[Ca^{2+}]_i$ levels during exposure to a toxic concentration of acetaminophen.

In liver cells, acetaminophen toxicity require cytochrome P450-mediated activation to NAPQI. This reactive metabolite conjugates with reduced glutathione and can lead to depletion of this protective thiol (28). Once glutathione levels fall below a critical level, the cell is vulnerable to the toxic effects of NAPQI (28). There is a lag period between exposure of liver cells to acetaminophen and the loss of cell membrane viability. This period results partly from the time it takes to deplete glutathione but also from events initiated by NAPQI, which take some time to kill the cell. Under the conditions of the present study, this lag period was >2 hr. During this 2-hr period, NAPQI initiates a toxic process that in some way results in damage to the cell membrane. This toxic process can be substantially ameliorated by antioxidants (22, 23) and sulfhydryl agents (24).

Toxic concentrations of acetaminophen have been found to increase glycogen phosphorylase *a* activity soon after exposure (10, 11, 17), and this was confirmed in the present study. Because an increase in $[Ca^{2+}]_i$ can activate phosphorylase *b* kinase via calmodulin (12), it has been proposed that this increase in phosphorylase activity results from a sustained increase in $[Ca^{2+}]_i$. However, the data in the present study indicate that were was no sustained increase in $[Ca^{2+}]_i$ during a 2-hr period before loss of cell membrane viability resulting from exposure to a toxic concentration of acetaminophen.

There could be a number of explanations for an increase in phosphorylase activity in the absence of a change in $[Ca^{2+}]_i$. cAMP bound to calmodulin can activate phosphorylase *b* kinase, with a resultant increase in phosphorylase *a* activity. However, others have failed to find such a change in cAMP after acetaminophen exposure (10), and we were unable to demonstrate any change in cAMP level during the 2-hr period before loss of cell membrane viability (data not shown). It is also known that respiratory toxins can enhance the rate of glycolysis by interfering with the rate of the phosphorylation-dephosphorylation equilibrium of the cell, resulting in activation of phosphorylase *a* (29). Such a response with respiratory toxins was demonstrated in the present study. Glycogen phosphorylase activity can also be enhanced by an increase in ADP or AMP or by a fall in ATP (12). These effects are not dependent on changes in $[Ca^{2+}]_i$. Acetaminophen toxicity in mouse hepatocytes is also associated with mitochondrial damage (19) and a fall in ATP (20). Acetaminophen itself is a reversible mitochondrial inhibitor (30), and NAPQI irreversibly damages the mitochondrial electron-transport chain (31). Hence, the increase in phosphorylase activity in the present study could be due to a decrease in ATP and concomitant increases in ADP and AMP. So the increase in phosphorylase *a* most likely results from this decreased energy supply to the cell and is not calcium related.

In mouse hepatocytes, acetaminophen toxicity involves oxidative injury. Studies from this laboratory have indicated that inhibition of the glutathione peroxidase/reductase enzyme system increases the toxic potential of acetaminophen (32). Furthermore, the resistance of hepatocytes from postnatal mice to acetaminophen toxicity is related to the higher activity of this enzyme system (33). Antioxidants such as vitamin E, *N,N'*-diphenyl-*p*-phenylenediamine, and promethazine protect cells from acetaminophen toxicity (23). These findings in the mouse are consistent with those in cultured rat hepatocytes, where it has been found that the toxicity of acetaminophen is dependent on a soluble source of ferric iron, can be prevented by addition of superoxide dismutase, catalase, or antioxidants, and can be enhanced by inhibition of glutathione reductase (3, 34, 35).

Using digital imaging fluorescence microscopy with single mouse hepatocytes, we have demonstrated that alterations in $[Ca^{2+}]_i$ do not play a role in the genesis of this oxidative injury. Indeed, the timing of any increase in $[Ca^{2+}]_i$ is such that it most likely results from an influx of extracellular calcium through the damaged cell membrane. The increase in $[Ca^{2+}]_i$ occurred shortly before loss of cell membrane patency and after indications of damage to the cell membrane (blebbing). Hence, it is unlikely that damaging events initiated by this rise in $[Ca^{2+}]_i$ could contribute to the lethal processes associated with acetaminophen toxicity that can occur within the first hour of incubation (as demonstrated in Fig. 3). Furthermore, when hepatocytes were incubated in calcium-free buffer after a 2-hr exposure to acetaminophen, there was no increase in $[Ca^{2+}]_i$ but the cells still died. Likewise, $[Ca^{2+}]_i$ does not appear to be involved in the blebbing that occurs on the cell membrane. Blebbing occurred on the cell surface of hepatocytes before the late rise in $[Ca^{2+}]_i$ and also occurred on cells incubated in calcium-free buffer, where there was no change in $[Ca^{2+}]_i$ before loss of membrane patency. Recent evidence indictes that a rise in $[Ca^{2+}]_i$ is not a prerequisite for bleb formation caused by oxidative stress or ATP depletion (36, 37).

It is difficult to reconcile the oxidative events associated with acetaminophen toxicity in mouse hepatocytes with those proposed for oxidative stress involving alteration in $[Ca^{2+}]_i$ (4). It is suggested that the latter involve oxidation of sulfhydryl groups on proteins critical for the maintenance of low $[Ca^{2+}]_i$, such as Ca^{2+}/Mg^{2+}-ATPase, leading to a sustained increase in $[Ca^{2+}]_i$. It is also proposed that this increase in $[Ca^{2+}]_i$ results in impaired mitochondrial function, protease and endonuclease activation, and perturbations in cytoskeletal organisation (2). However, by using a more direct method of measuring $[Ca^{2+}]_i$ in single cells during acetaminophen intoxication, we have found that $[Ca^{2+}]_i$ does not change during the time when critical toxic events take place.

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