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Regulation of intracellular calcium compartmentation: Studies with isolated hepatocytes and *t*-butyl hydroperoxide

(mitochondrial Ca^{2+}/extramitochondrial Ca^{2+}/NADPH oxidation/thiol homeostasis/arsenazo III)

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ABSTRACT In suspensions of isolated hepatocytes, two intracellular Ca^{2+} pools were distinguished in the presence of the metallochrome indicator arsenazo III, first by treatment with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and then with the Ca^{2+} ionophore A23187. The available evidence indicates that the two pools are of mitochondrial and extramitochondrial origin. Metabolism of *t*-butyl hydroperoxide by hepatocytes caused release of Ca^{2+} from both compartments concomitant with oxidation of cellular glutathione and NADPH, which was followed by characteristic alterations in cell surface structure. When NADPH oxidation was prevented by selective inactivation of glutathione reductase, *t*-butyl hydroperoxide metabolism was without effect on the mitochondrial Ca^{2+} pool, whereas the loss from the extramitochondrial pool was accelerated. Our results suggest that different regulatory mechanisms modulate mitochondrial (NADPH-dependent) and extramitochondrial (thiol-dependent) Ca^{2+} compartmentation and that disturbance of normal Ca^{2+} homeostasis may be critical in peroxide-induced cytotoxicity.

The calcium ion exerts a profound influence on a wide variety of cellular processes (1–3). The distribution of Ca^{2+} within the cell is complex, involving the binding to cellular components (4, 5) as well as the action of specific compartmentation processes (6). The cytosolic Ca^{2+} concentration is maintained at levels 3–4 orders of magnitude lower than in the extracellular medium due to active extrusion of the ion by specific Ca^{2+} pumps present in the plasma membrane as well as the interdependent action of intracellular sequestration sites (7). However, at the present time little is known about the physiological mechanisms involved in intracellular Ca^{2+} homeostasis.

Several methods have been proposed to study Ca^{2+} fluxes from one cellular compartment to another. Claret-Berthon *et al.* (8), using $^{45}Ca^{2+}$ loading in the perfused rat liver, proposed a model with three different calcium pools: a dynamic cytoplasmic pool (which can itself be subdivided into mitochondrial, microsomal, and cytosolic subfractions), a slowly exchangeable nuclear pool, and an apparently nonexchangeable mitochondrial pool (insoluble calcium). In isolated cells, compartmentation and fluxes of Ca^{2+} have been studied by the use of (*i*) calcium-selective microelectrodes (9); (*ii*) photoproteins, such as aequorin (10); (*iii*) metallochromic indicators, notably arsenazo III (11); and (*iv*) rapid cell disruption techniques with measurement of Ca^{2+} content in the different organelle fractions (12). Recently, Murphy *et al.* (13) developed a method for the quantitation of cytosolic free Ca^{2+} using arsenazo III to measure Ca^{2+} fluxes through the hepatocyte plasma membrane which was made permeable by digitonin treatment.

Data obtained by these methods indicate that the mitochondria play a major role in the control of calcium compartmentation and the regulation of cytosolic Ca^{2+}. It has been demonstrated by Lehninger *et al.* (14), and subsequently confirmed by others (15–17), that the pyridine nucleotide redox state is a determining factor in the ability of mitochondria to take up and retain Ca^{2+}. In addition, Sies *et al.* (18) recently have shown that the oxidation of cytosolic pyridine nucleotides can cause a release of Ca^{2+} from isolated perfused liver, although the site(s) from which Ca^{2+} was released could not be identified.

In the present work we introduce a nondisruptive method for measuring two distinct intracellular Ca^{2+} pools—mitochondrial and extramitochondrial. Using this system, we have observed that significant alterations in hepatocyte Ca^{2+} homeostasis are caused by *t*-butyl hydroperoxide metabolism. Hydroperoxides, known to cause oxidative stress and toxicity in various tissues (cf. 19, 20), are metabolized in hepatocytes by the glutathione peroxidase system (15), leading to glutathione (GSH) and NADPH oxidation. The metabolism of *t*-butyl hydroperoxide has been shown to impair the ability of liver mitochondria to retain Ca^{2+} (15–17) and to cause Ca^{2+} release from perfused liver (18). Our results indicate that different regulatory mechanisms control the mitochondrial and extramitochondrial compartments and that the metabolism of *t*-butyl hydroperoxide causes Ca^{2+} loss from both intracellular pools. Depletion of cell Ca^{2+} is associated with characteristic alterations in hepatocyte surface structure, which may be an early indication of cytotoxicity.

MATERIALS AND METHODS

Materials. Collagenase (grade II) and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) were obtained from Boehringer Mannheim. Arsenazo III, bovine serum albumin (fraction V), Hepes, NADH, and ruthenium red were purchased from Sigma. The cation ionophore A23187 was purchased from Calbiochem-Behring and Percoll was obtained from Pharmacia. BCNU [*N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea] was a gift from Jakob Kaluski of Bristol Laboratories (Stockholm, Sweden). All other reagents were commercial products of the highest available grade of purity.

Hepatocyte Isolation and Incubation. Hepatocytes were isolated from male Sprague–Dawley rats (180–200 g; allowed food and water ad lib) by collagenase perfusion of the liver (21). The yield was 2–4 × 10^8 cells per liver and, immediately after isolation, the hepatocytes excluded both trypan blue and NADH (90–100%) (cf. 21). Hepatocytes were incubated in Krebs–Henseleit buffer at pH 7.4, supplemented with 12.6 mM

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Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; BCNU, *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea; GSH, glutathione; GSSG, glutathione disulfide.

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Hepes (22), at a concentration of 6×10^6 cells per ml for Ca^{2+} experiments or 1×10^6 cells per ml for GSH and NADPH assays. After a 15-min preincubation period at 37°C, samples were taken as zero time and *t*-butyl hydroperoxide was added. Special pretreatments of the hepatocytes were carried out as follows: (*i*) To protect the cells from thiol group oxidation during *t*-butyl hydroperoxide metabolism, hepatocytes were preincubated for 5 min with 2 mM 1,4-dithiothreitol. (*ii*) To inhibit mitochondrial Ca^{2+} uptake (23), samples of hepatocytes were incubated in the Krebs–Henseleit medium (described above) for 30 min at 37°C in the presence of either 5 or 20 μM of ruthenium red; this treatment did not affect the viability of the hepatocytes. (*iii*) To inactivate glutathione reductase [NAD(P)H:oxidized-glutathione (GSSG) oxidoreductase, EC 1.6.4.2], hepatocytes first were preincubated in an amino acid–supplemented Krebs–Henseleit medium in the presence of 100 μM BCNU as described by Eklöw *et al.* (24). After 30 min the cells were washed, resuspended, and incubated for an additional 90 min in the same medium, except that BCNU was absent. This procedure provides hepatocytes with long-lasting inhibition of glutathione reductase, but with a normal cellular GSH level at the end of the treatment (24).

Biochemical Assays. For the measurement of cellular Ca^{2+}, the hepatocytes were separated from the Ca^{2+}-containing Krebs–Henseleit medium by rapid centrifugation through a suspension of Ca^{2+}- and Mg^{2+}-free Hanks' solution (13) and Percoll (final density: 1.06 g/ml). They then were quickly resuspended in the modified Hanks' medium and were separated into two parts; one part was used for counting the number of cells present and assaying cell viability (21), and the other part was used for Ca^{2+} measurement. Centrifugation of the cells through the Percoll mixture removes nonviable cells, ensuring that, even at the higher dose of *t*-butyl hydroperoxide, only viable cells were assayed. In all cases but one (see *Results*) cell viability remained quite high (>75%) throughout the 30-min incubation period.

Intracellular Ca^{2+} content was determined by dual wavelength spectrophotometry (685–675 nm) by using an Aminco DW2 UV/VIS spectrophotometer and purified arsenazo III (2,2'-[1,8-dihydroxy-3,6-disulpho-2,7-naphthalene-bis(azo)]-dibenzenearsonic acid) (11) (final concentration: 30 μM). FCCP (10 μM) was added first to the cell suspension ($\approx 4 \times 10^6$ cells per ml) and Ca^{2+} release was recorded until no further change in absorbance was observed. At this point, the Ca^{2+} ionophore A23187 (15 μM) was added and Ca^{2+} release was recorded.

In experiments with digitonin-permeabilized hepatocytes, the cells were carried through the procedure just described after preincubation for 30 min with or without 20 μM ruthenium red. Various concentrations of Ca^{2+} (up to 5 μM) were added; this was followed by the addition of 2 μM digitonin. After Ca^{2+} uptake was complete, 10 μM FCCP was added and subsequent release was recorded.

Hepatocyte GSH was measured either by the colorimetric assay of Saville (25) or by HPLC as described by Reed *et al.* (26). Cellular pyridine nucleotide levels were determined spectrophotometrically as described by Klingenberg (27).

Scanning Electron Microscopy. Samples were processed by standard procedures, which involved glutaraldehyde and osmium fixation followed by critical point drying. A Jeol model JSM35 scanning electron microscope was used to visualize and photograph the samples. A large number of hepatocytes were examined and representative cells showing typical morphology were photographed.

RESULTS

A typical pattern of Ca^{2+} release caused by the addition of FCCP and the ionophore A23187 to isolated hepatocytes is illustrated in Fig. 1*A*. The use of higher concentrations of FCCP did not result in any increased Ca^{2+} release over what was observed with the 10 μM concentration. Administration of the ionophore A23187 was found to cause complete Ca^{2+} release (equal to the total release caused first by FCCP and then by the ionophore), and there was no further release upon subsequent addition of FCCP. The FCCP-releasable and FCCP-nonreleasable cellular Ca^{2+} pools constitute approximately 60% and 40%, respectively, of the total amount of Ca^{2+} that could be released from control hepatocytes incubated under our experimental conditions; this proportion was quite consistent from one cell preparation to another.

A variety of experiments were undertaken to characterize these two Ca^{2+} pools. Administration of the uncoupler dicumarol (30 μM) produced the same Ca^{2+} release as FCCP and did not affect the Ca^{2+} release caused by subsequent addition of A23187 (not shown). Preincubation of hepatocytes with ruthenium red to block the uptake portion of normal mitochondrial Ca^{2+} cycling caused a substantial change in the proportions of the pools; this pretreatment resulted in a dose-dependent decrease in the size of the FCCP-releasable Ca^{2+} pool as well as a lesser decrease in the total level of releasable Ca^{2+} (Fig. 1 *B* and *C*). Hepatocytes that were preincubated in 20 μM ruthenium red and then permeabilized by digitonin treatment took up none of the Ca^{2+} added to the extracellular medium (up to 5 μM). However, when control cells were permeabilized

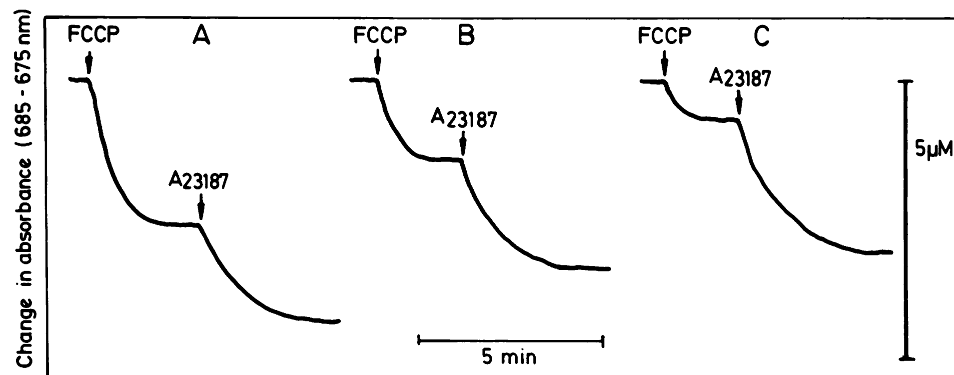

FIG. 1. Calcium release from hepatocytes by FCCP and by A23187. Cells were incubated and prepared for spectrophotometry as described. At the times shown, 10 μM FCCP or 15 μM A23187 was added, and traces typical of those shown were recorded. For the Ca^{2+}/arsenazo III experiments, $\Delta A_{685-675} = 0.005$ per 1 μM Ca^{2+}. (*A*) Control incubation; (*B*) preincubation of hepatocytes with 5 μM ruthenium red; (*C*) preincubation of hepatocytes with 20 μM ruthenium red. Traces are representative of results obtained with five or more separate cell preparations.

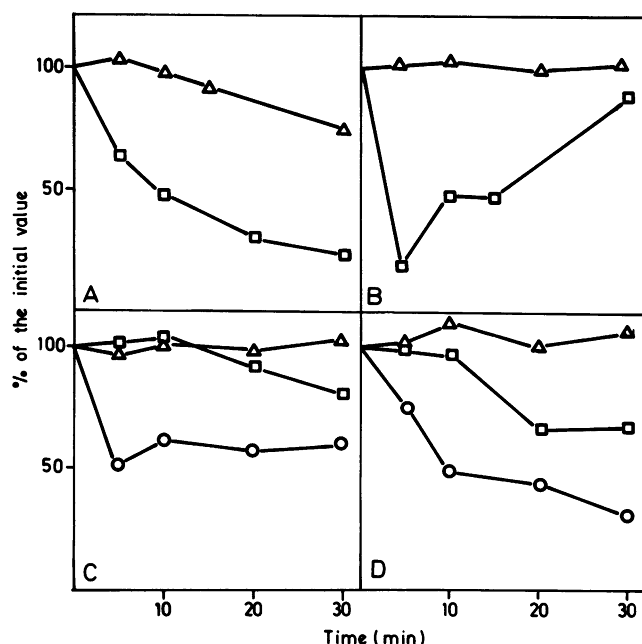

FIG. 2. Changes induced by the metabolism of *t*-butyl hydroperoxide in the levels of cellular GSH (*A*), NADPH (*B*), FCCP-releasable Ca^{2+} (*C*), and FCCP-nonreleasable Ca^{2+} (*D*). Cells were incubated as control (△) or with *t*-butyl hydroperoxide (□, 1 mM; ○, 4 mM). The results are expressed as percentage of the values at time zero and are typical of three to five trials. The absolute values at this time per 1 × 10^6 cells were: GSH, 45.2 nmol; NADPH 2.8 nmol; FCCP-releasable Ca^{2+}, 1.8 nmol; FCCP-nonreleasable Ca^{2+}, 1.1 nmol.

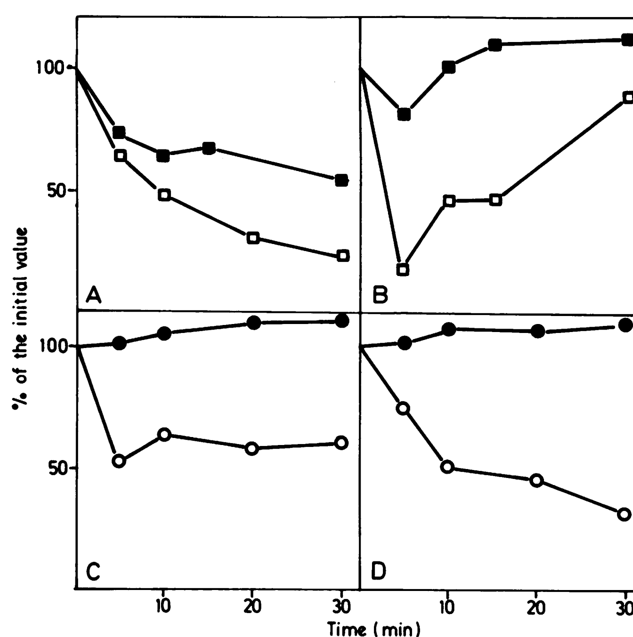

FIG. 3. Effects of 1,4-dithiothreitol on changes induced by the metabolism of *t*-butyl hydroperoxide in the levels of cellular GSH (*A*), NADPH (*B*), FCCP-releasable Ca^{2+} (*C*), and FCCP-nonreleasable Ca^{2+} (*D*). Cells were incubated as control (open symbols) or with 2 mM 1,4-dithiothreitol (closed symbols) before the addition of *t*-butyl hydroperoxide (□, ■, 1 mM; ○, ●, 4 mM) at time zero. The results are expressed as percentage of the values at time zero and represent typical values for three to five experiments. The absolute values at this time were not different from those given in Fig. 2, for both control and 1,4-dithiothreitol-treated hepatocytes.

with digitonin in the presence of extracellular Ca^{2+}, rapid Ca^{2+} uptake ensued; subsequent addition of FCCP then caused complete release of the Ca^{2+} taken up (not shown). This evidence indicates that the FCCP-releasable Ca^{2+} may be of mitochondrial origin.

In intact hepatocytes, *t*-butyl hydroperoxide is metabolized by the GSH peroxidase (GSH:H_2O_2 oxidoreductase, EC 1.11.1.9) system present both in the cytosolic and mitochondrial compartments (15). This results in the formation of GSSG, which is subsequently reduced to GSH by glutathione reductase at the expense of NADPH. Under our experimental conditions, the metabolism of *t*-butyl hydroperoxide by control hepatocytes led to both a marked decrease in cellular GSH level (Fig. 2*A*) and a sharp initial drop in NADPH concentration (Fig. 2*B*). No change in NADH level occurred (not shown). Incubation of hepatocytes with *t*-butyl hydroperoxide also affected the size of both Ca^{2+} pools; the size of the FCCP-releasable pool was markedly affected only at the higher concentration of the hydroperoxide (Fig. 2*C*), whereas the size of the FCCP-nonreleasable Ca^{2+} pool was affected by both concentrations in a dose- and time-dependent manner (Fig. 2*D*).

To study the relationship between the peroxide-associated oxidation of GSH and NADPH and the decrease in the Ca^{2+} pools, two series of experiments were performed. Cellular thiol groups were protected from oxidation during *t*-butyl hydroperoxide metabolism by the addition of 1,4-dithiothreitol to the incubation 5 min prior to addition of the hydroperoxide. This pretreatment prevented the effects of *t*-butyl hydroperoxide on GSH and NADPH levels and on the loss of Ca^{2+} from both pools (Fig. 3). In another group of experiments, hepatocytes were pretreated with BCNU, a selective inactivator of glutathione reductase (28), to minimize the NADPH oxidation that occurs during hydroperoxide metabolism. With this treatment GSH depletion was accelerated (as reduction of the GSSG formed was prevented) and could be separated from NADPH oxidation (Fig. 4 *A* and *B*). The responses of the two Ca^{2+} pools to these conditions also could be distinguished; the BCNU pretreatment reversed the hydroperoxide-induced loss from the mitochondrial pool, whereas the extramitochondrial pool became more rapidly and extensively depleted (Fig. 4 *C* and *D*). Inhibition of glutathione reductase also enhanced dramatically the toxicity of *t*-butyl hydroperoxide, because after only 5 min of exposure to the hydroperoxide the number of viable cells was insufficient to continue Ca^{2+} measurement (see *Materials and Methods*).

Perturbation of the hepatocyte surface structure, with characteristic blebbing of the plasma membrane, was an early sign of *t*-butyl hydroperoxide toxicity (Fig. 5*A*) occurring within 30 min of hydroperoxide addition and well before any significant loss of cell viability. As shown in Fig. 5*B*, this effect was prevented by the presence of 1,4-dithiothreitol in the incubation medium, whereas it was markedly enhanced in the BCNU-pretreated hepatocytes (Fig. 5*C*). Therefore, this loss of normal surface morphology appears to be related to the thiol group oxidation and depletion of cell Ca^{2+} that results from hydroperoxide metabolism.

DISCUSSION

In this work we have employed a relatively simple method to quantitate two different intracellular calcium pools in isolated hepatocytes. The FCCP-releasable Ca^{2+} pool appears to be derived from the mitochondrial compartment, measurable as a consequence first of collapse of the mitochondrial membrane and then a transient release of Ca^{2+} into the cytosol and subsequent extrusion from the cell. With isolated, coupled mitochondria, the collapse of the proton electrochemical gradient by uncouplers (such as FCCP and dicumarol) has been shown

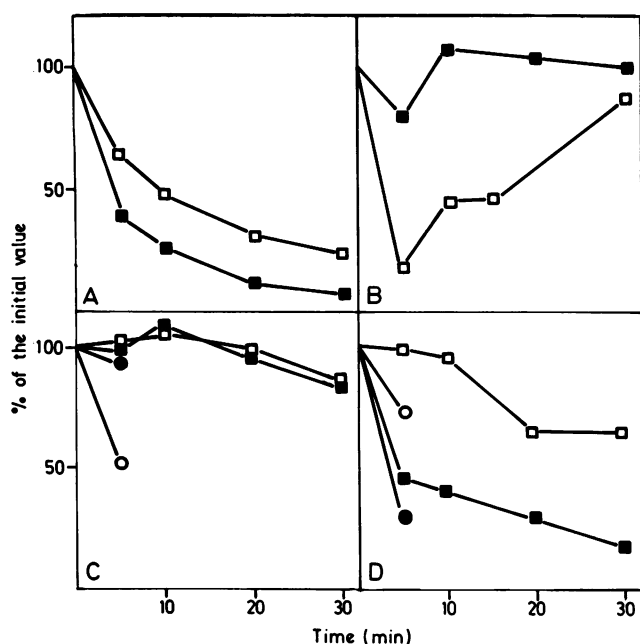

FIG. 4. Effects of BCNU on changes induced by the metabolism of *t*-butyl hydroperoxide in the levels of cellular GSH (*A*), NADPH (*B*), FCCP-releasable Ca^{2+} (*C*), and FCCP-nonreleasable Ca^{2+} (*D*). Cells were pretreated as control (open symbols) or with BCNU (closed symbols), as described, prior to the addition of hydroperoxide (□, ■, 1 mM; ○, ●, 4 mM) at time zero. The results are typical of values obtained for three to five trials and are expressed as percentage of the time zero value. The absolute values for the BCNU-pretreated hepatocytes at this time per 1 × 10^6 cells were: GSH, 52.5 nmol; NADPH, 3.5 nmol; FCCP-releasable Ca^{2+}, 1.7 nmol; FCCP-nonreleasable Ca^{2+}, 1.0 nmol.

to cause Ca^{2+} release (29). In addition, the amount of Ca^{2+} released from isolated hepatocytes by FCCP as measured with arsenazo III is very close to the mitochondrial Ca^{2+} level determined by atomic absorption spectrometry after rapid cell disruption (13). The Ca^{2+} that is releasable by both FCCP and dicumarol in our system is in close agreement with these measurements.

Further, our findings show that incubation with ruthenium red, which inhibits only mitochondrial Ca^{2+} uptake (22, 30), leads to a substantial decrease in the size of the FCCP-releasable pool as well as a reduction in total cell Ca^{2+}, indicating that the plasma membrane pumps extrude Ca^{2+} that is lost by the mitochondria. Results obtained from the addition of digitonin and FCCP to ruthenium red-pretreated hepatocytes also indicate that the uncoupler's releasing action is specific for the mitochondrial compartment. Moreover, studies done with rat liver microsomes suggest that the endoplasmic reticulum [which is thought to sequester a large majority of the cell's nonmitochondrial Ca^{2+} (13)] is unaffected by uncouplers such as FCCP and dicumarol but is highly sensitive to A23187 (unpublished results). Taken together, these facts provide strong support for the mitochondrial origin of the FCCP-releasable Ca^{2+} pool.

The amount of Ca^{2+} that is releasable from isolated hepatocytes by the cation ionophore A23187 is higher than that releasable by FCCP alone and is also higher than the mitochondrial Ca^{2+} content as determined by atomic absorption spectrometry (13, 29, 31, 32). The ionophore alone releases the same total amount of Ca^{2+} from hepatocytes as does sequential FCCP/A23187 treatment and also readily induces Ca^{2+} release from isolated microsomes. Therefore, it seems justified to conclude that the FCCP-releasable and FCCP-nonreleasable Ca^{2+} pools in this study represent Ca^{2+} derived from the mitochondrial and extramitochondrial compartments, respectively.

The observed decrease in the mitochondrial Ca^{2+} pool during *t*-butyl hydroperoxide metabolism is in agreement with the results previously obtained with isolated mitochondria by our group (17) and others (15). Our results also support the hypothesis that the oxidation of mitochondrial pyridine nucleotides (primarily NADPH) is responsible for this decrease in mitochondrial Ca^{2+} during hydroperoxide metabolism. Thus, the inhibition of both pyridine nucleotide oxidation and mitochondrial Ca^{2+} release by 1,4-dithiothreitol and BCNU indicates that the factor responsible for the decrease in mitochondrial Ca^{2+} level is distal to glutathione reductase. Therefore, our data confirm the role of the pyridine nucleotide redox state in the control of mitochondrial Ca^{2+} concentration in the intact cell, as predicted by Lehninger *et al.* (14).

Incubation of rat hepatocytes with the alkylating agent BCNU has been shown to cause inactivation of glutathione reductase (28), and Eklöw *et al.* have recently developed an incubation procedure that yields hepatocytes with inhibited glutathione reductase but that exhibit a normal GSH level (24). Incubation of hepatocytes, pretreated with BCNU according to this procedure, with *t*-butyl hydroperoxide resulted in enhanced loss of cellular GSH and FCCP-nonreleasable Ca^{2+} without apparent effects on the pyridine nucleotide redox level or the size of the FCCP-releasable Ca^{2+} pool (cf. Fig. 4). Therefore, it appears that oxidation of GSH, rather than pyridine nucleotide oxidation, is responsible for the release of extramitochondrial Ca^{2+} from the hepatocytes. A similar effect of GSH

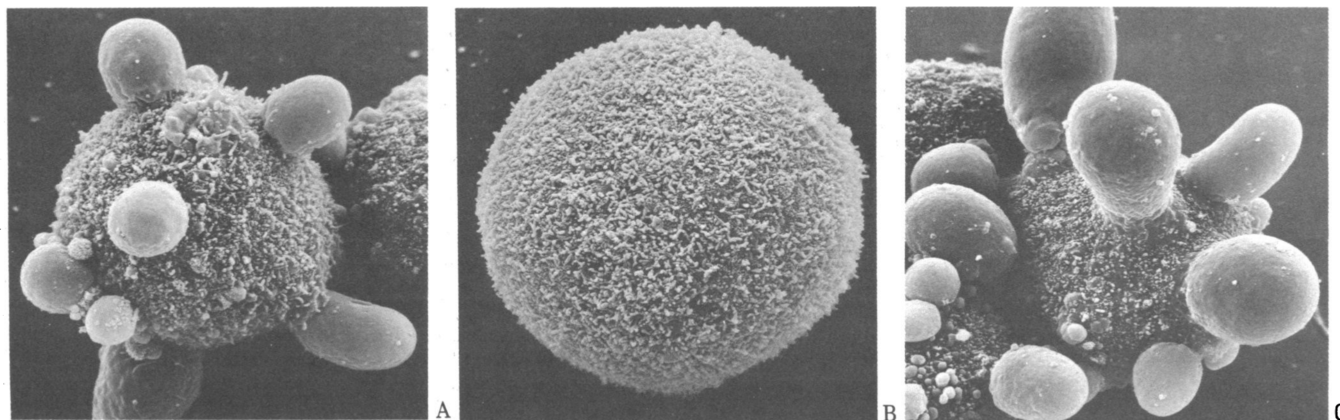

FIG. 5. Scanning electron micrographs of treated hepatocytes. Micrographs were taken 30 min after the addition of 4 mM *t*-butyl hydroperoxide (*A*; ×2,100), 4 mM *t*-butyl hydroperoxide and 2 mM 1,4-dithiothreitol (*B*; ×2,800), or 1 mM *t*-butyl hydroperoxide and 100 μM BCNU (*C*; ×2,100).

depletion on cellular calcium homeostasis has recently been observed with several other agents that affect the GSH status of isolated hepatocytes (33).

Cumulative evidence supports the assumption that the perturbation of hepatocyte surface structure during *t*-butyl hydroperoxide metabolism is related to GSH depletion and loss of Ca^{2+} from the extramitochondrial compartment (33). Soluble thiols, notably GSH, may exert a protective effect by (*i*) preventing the inactivation of Ca^{2+}-binding proteins such as calmodulin, which has functionally important methionine groups (34), and (*ii*) by maintaining the thiol groups of intracellular membrane proteins in their normal state, most probably via the cytosolic thiol transferase (35). The latter may be particularly important for the maintenance of the calcium sequestering function of the endoplasmic reticulum (36). The destruction of calcium sequestration in the endoplasmic reticulum would cause a gross disturbance of intracellular Ca^{2+} homeostasis and the inhibition of enzymes associated with the hepatocyte cytoskeletal apparatus, such as actomyosin ATPase. The link between intracellular thiol and Ca^{2+} homeostasis and the functional operation of the hepatocyte cytoskeletal apparatus awaits further investigation.

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