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Intracellular Ca^{2+} Thresholds That Determine Survival or Death of Energy-Deprived Cells

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Increase of intracellular ionized or free Ca^{2+} is thought to play a central role in cell death due to ATP depletion. However, concurrently operative mechanisms of injury that do not require intracellular Ca^{2+} increases have made it difficult to test this hypothesis or to determine the concentrations at which intracellular Ca^{2+} becomes lethal. The predominant Ca^{2+}-independent mechanism of injury during ATP depletion involves the loss of cellular glycine. This type of damage can be fully inhibited by adding the amino acid exogenously. Using glycine to suppress Ca^{2+}-independent plasma membrane damage, we have examined the effect of intracellular Ca^{2+} elevations on cell viability during ATP depletion. Madin-Darby canine kidney (MDCK) cells were depleted of ATP by incubation with a mitochondrial uncoupler in glucose-free medium. Free Ca^{2+} concentration in the medium was varied between 26 nmol/L and 1.25 mmol/L in the presence of a Ca^{2+} ionophore. Measurements with the Ca^{2+} probes fura-2, furaptra, and fura-2FF showed that intracellular Ca^{2+} was clamped at extracellular levels under these conditions. Cell survival during ATP depletion was indicated by viable cells recovered 24 hours later. The results show that ATP-depleted cells can sustain high levels of intracellular Ca^{2+} (100 μmol/L) for prolonged periods and remain viable if plasma membrane damage is prevented by glycine. Cell death was observed only when intracellular free Ca^{2+} was allowed to increase beyond 100 μmol/L, and this was associated with dramatic nuclear alterations: chromatin condensation, loss of nuclear lamins, and breakdown of DNA into large 50- to 150-kb fragments. Our studies demonstrate unexpectedly high resistance of cells to calcium cytotoxicity if glycine that is lost during ATP depletion is restored. In addition, they provide insights into novel mechanisms of nuclear disintegration and DNA damage that are triggered when the high thresholds of intracellular Ca^{2+} required for cell death are exceeded. ***(Am J Pathol 1998, 152:231–240)***

Marked decline of adenosine triphosphate (ATP) is a universal response of mammalian cells to oxygen deprivation during clinically important conditions such as ischemia.[1–10] In large part, this is caused by cell membranes that are leakier to ions than membranes in hypoxia-tolerant organisms.[1] The associated failure of energy-dependent ion pumps leads to loss of cellular ion homeostasis, including that of Ca^{2+}.[1–11] Elevation of intracellular ionized or free Ca^{2+} (hereafter referred to as Ca_f) above the normal basal level of $\sim 10^{-7}$ mol/L may derive from both influx and release from intracellular stores.[12–16] Increases of Ca_f are physiological during signal transduction in energy-replete normal cells,[17,18] but they must be transient; otherwise, Ca^{2+} may act as a cellular toxin.[17–20] It is generally held that elevations of Ca_f trigger a number of degenerative biochemical and structural events in energy-depleted cells also and thereby cause cell death.[1–10] However, injury mechanisms that do not require increases of Ca_f operate concurrently in energy-deprived cells. This has made it impossible to test the Ca^{2+} cytotoxicity hypothesis or to determine the concentrations at which Ca^{2+} is lethal.[2–4,13,21,22]

The predominant Ca^{2+}-independent mechanism of injury during ATP depletion involves the loss of cell-associated glycine (for reviews, see Refs. 2 and 22). Thus, provision of glycine protects against cellular injury under various conditions of ATP depletion. This has been demonstrated in diverse kinds of cells subjected to hypoxia, ischemia, or metabolic inhibition.[2,13,22–28] Glycine is normally transported against steep gradients to high concentrations (5 to 20 mmol/L) within cells but is lost to the extracellular milieu during energy deprivation.[22] Loss of glycine leads to plasma membrane damage, leakage of cytosol, and necrosis.[22–28] This type of damage takes place even when Ca_f is not allowed to increase above 10^{-7} mol/L[13] and is prevented by the addition of glycine, which diffuses into cells.[2,13,22–28] Based on these obser-

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vations, we used glycine to suppress Ca^{2+}-independent plasma membrane damage during ATP depletion and have directly and quantitatively examined for the first time the role played by Ca_f increases in the survival or death of cells after this common type of injury.

Materials and Methods

ATP Depletion, Ca_f Clamping, and Measurement of Cell Survival

Madin-Darby canine kidney (MDCK) cells were cultured, plated at 0.4×10^6 per 35-mm dish, and used after overnight growth. ATP depletion was initiated by incubating cells in glucose-free Krebs-Ringer bicarbonate buffer (KRB) containing 15 μmol/L carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), a mitochondrial uncoupler. Free Ca^{2+} in the buffer was adjusted to concentrations between 26 nmol/L and 1.25 mmol/L by adding EGTA.[29] Ionomycin (5 μmol/L), a Ca^{2+} ionophore, was included in the buffer so that cells were permeabilized to Ca^{2+}. Experiments were done with or without the addition of 5 mmol/L glycine and/or 4% sucrose, a membrane-impermeant osmolyte, to the incubation medium. After 3 hours of incubation, the integrity of plasma membranes was assessed by measuring lactate dehydrogenase (LDH) released from cells into the medium.[13] Retention by cells of LDH (molecular weight 136,000) reflected their ability to exclude the much smaller vital dye propidium iodide (molecular weight 668) and thus provided a reliable index of membrane integrity.[30] Cells were then returned to full culture medium (Dulbecco's modified Eagle's medium with 25 mmol/L glucose, 2 mmol/L glutamine, 10% calf serum), and 24 hours later, the cells were trypsinized, and those excluding trypan blue were counted with a hemacytometer.

Measurement of Ca_f

Ca_f was quantitated fluorometrically with the low-affinity Ca^{2+} indicators furaptra and fura-2FF (Teflabs, Austin, TX). The latter is a new Ca^{2+} indicator, with little or no binding by Mg^{2+}. Similar results were obtained with both indicators, and only those with fura-2FF are shown. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), a nonfluorescent analog of CCCP, was used to deplete cells of ATP. MDCK cells were grown overnight on coverslips and loaded for 1 hour in KRB containing 5 μmol/L fura-2FF, at 37°C. Coverslips with attached cells were then placed in a perfusion cuvette of a spectrofluorometer with temperature control. The cells were perfused sequentially with buffers containing FCCP and ionomycin and different concentrations of free Ca^{2+}, adjusted by EGTA. The buffers also contained 4% sucrose to prevent cell swelling and leakage of dye. Experiments were done with or without 5 mmol/L glycine in the perfusion medium. Measurements of Ca_f in ATP depleted cells without glycine (Figure 2c) or after withdrawal of glycine (Figure 2d) were done within 1 hour of glycine free perfusion, well before plasma membrane integrity was compromised due to absence of the amino acid. To study the reversibility of Ca_f increase, Ca_f was first allowed to rise to desired levels using ionomycin, followed by perfusion with a solution containing the same concentration of $Ca,^{2+}$ 25 mmol/L glucose, and 2 mmol/L glutamine but no ionomycin. Separate studies showed that provision of substrates in this manner led to the generation and increase of cell ATP after a brief lag period (not shown). At the end of every experiment, a calibration was performed and Ca_f was calculated by the Grynkiewicz equation.[31] For the calculation, we used the K_d values (50 μmol/L for furaptra and 25 μmol/L fura-2FF) provided by Teflabs. Nevertheless, we also determined the K_d values independently in ATP-depleted cells by the *in situ* method.[32–35] The calculated K_d values for Ca^{2+}-indicator complexes were not different from that provided by the manufacturer.

Examination of Ca_f Distribution

MDCK cells were grown overnight on coverslips and loaded for 90 minutes in KRB containing 20 μmol/L Calcium Green-5N acetoxymethyl ester ($K_d \approx 3.3$ μmol/L; Molecular Probes, Eugene, OR). After 40 minutes of incubation in KRB containing FCCP, ionomycin, and glycine with 100 nmol/L or 100 μmol/L Ca^{2+}, coverslips were inverted over shallow chambers filled with incubation buffer. Optical sections of 0.72 μm in thickness were visualized at 488 nm excitation and 520 nm emission on Zeiss laser scanning confocal microscope.

Electron Microscopy

After 3 hours of treatment, the incubation medium was saved to measure free LDH, and cells were fixed in 2% glutaraldehyde with 50 mmol/L lysine, 50 meq of Na, and 100 meq of cacodylic acid[36] and subsequently processed for electron microscopy.

Measurement of DNA Strand Breaks

DNA strand breaks were measured by alkaline unwinding assay.[37] After incubation for 3 hours with CCCP and ionomycin in the presence of glycine and sucrose (see above), cells were lysed and exposed to alkali (pH 12.6) for 30 minutes at 15°C. Ethidium bromide was then added to measure the fluorescence at 520 nm excitation and 590 nm emission. Under these conditions, ethidium bromide fluorescence is strictly dependent on preferential binding to double-stranded DNA. Formation of strand breaks increases the rate of DNA unwinding in alkali and reduces ethidium bromide fluorescence. The percentage of residual double-stranded DNA after alkali exposure was calculated by the equation: $\%D = 100(F(P) - F(B))/(F(T) - F(B))$, where $F(P)$, $F(T)$, and $F(B)$ are the fluorescence values for sample, total, and background, respectively.

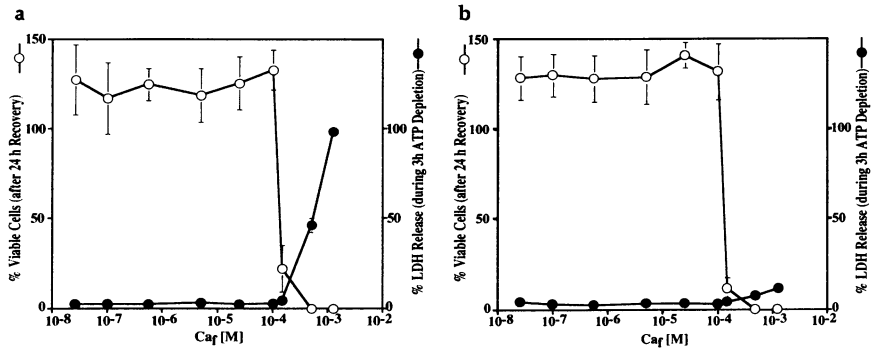

Figure 1. Ca_f levels that determine survival or death of ATP-depleted cells. MDCK cells were depleted of ATP for 3 hours in the presence of 5 mmol/L glycine (**a**) or 5 mmol/L glycine plus 4% sucrose (**b**). Neither glycine nor sucrose affected the extent of ATP depletion (not shown). Ca_f was clamped at desired levels (26 nmol/L to 1.25 mmol/L), using ionomycin and EGTA. At the end of ATP depletion, LDH released from the cells into the incubation medium was assessed. The cells were then transferred back to culture medium. Cell survival during ATP depletion is indicated by viable cells recovered 24 hours later, expressed as a percentage of cell number in culture dishes immediately before the experiment (mean ± SE; n = 4). During the same period, sham-incubated controls not subjected to ATP depletion proliferated normally (261 ± 20% viable cells after 24 hours, not shown). Thus, cells with ≤100 μmol/L Ca_f survived but were growth inhibited during the 24-hour recovery period relative to controls. Nevertheless, they were functional, divided subsequently after the initial delay, and proliferated normally over the next several days (not shown). All cells incubated under conditions identical to those described above, but without glycine, released LDH and did not survive (not shown).

Electrophoretic Analysis of DNA Breakdown

DNA fragments were visualized by both conventional agarose gel electrophoresis[38] and field inversion gel electrophoresis (FIGE).[39] After 3 hours of treatment, the incubation medium was saved to measure free LDH, and cells were lysed in hypotonic lysis buffer (0.5% Triton X-100, 10 mmol/L Tris, 20 mmol/L EDTA, pH 7.4). Lysate was centrifuged at 14,000 × *g* for 20 minutes to separate broken DNA from intact chromatin. After treatment with RNase A and proteinase K, DNA fragments in the supernatant were precipitated with ethanol and subjected to conventional agarose gel electrophoresis or FIGE. FIGE was accomplished on 1% agarose gel in 90 mmol/L Tris/acetate, 1 mmol/L EDTA buffer (pH 8.4). Runs were carried out at 20°C for 24 hours at a voltage gradient of 5 V/cm using switch times ramped from 1 to 25 seconds with a forward/reverse ratio of 3:1. A λ ladder PFG marker (New England Biolabs, Beverly, MA) was run in parallel to identify the sizes of DNA fragments. Gels were then stained with ethidium bromide and photographed.

Detection of Lamin Degradation

Lamins A and C were detected by immunoblotting using a monoclonal antibody (American Research Products, Belmont, MA). After experimental incubation, cells were collected and sedimented at 1000 × *g* for 10 minutes. Cell pellets were then solubilized in a sample buffer containing 6 mol/L urea, 2% SDS, 5% β-mercaptoethanol, and 62.5 mmol/L Tris (pH 6.8). Proteins in whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis, blotted on to polyvinylidene difluoride membranes, probed with the anti-lamin A/C antibody, and visualized by horseradish-peroxidase-labeled secondary antibodies.

Statistics

Values reported are means ± SE. Data were analyzed by analysis of variance for repeated measure designs.

Results

Exposure to the mitochondrial uncoupler CCCP in glucose free medium led to the decrease of ATP in MDCK cells to levels <1% of control values. The rates of decline were similar with or without glycine, as we have reported previously.[30] Likewise, ATP declined equivalently in cells with or without sucrose (not shown).

Effect of Ca_f Levels on Survival of ATP-Depleted Cells

In the presence of ionomycin and defined medium Ca^{2+} concentrations, intracellular Ca^{2+} (Ca_f) equilibrated rapidly with extracellular Ca^{2+} and was therefore clamped at these levels (Figure 2; see next section for technical issues). After 3 hours, the cells so treated were returned to full culture medium. Cell survival during ATP depletion was indicated by viable cells recovered 24 hours later.

Without glycine supplementation, ATP-depleted cells developed plasma membrane damage regardless of the presence or absence of sucrose at every Ca^{2+} concentration tested. This was indicated by release of LDH from cells into the medium (92.4 ± 1.9% of total LDH; n = 21) and is consistent with the known requirement of glycine to maintain plasma membrane integrity during ATP depletion of cells *in vitro*.[2,13,15,22–28] As expected, no cells survived (not shown). However, when 5 mmol/L glycine was provided, all groups of cells exposed to 100 μmol/L Ca^{2+} or less retained LDH and survived when allowed to recover (Figure 1a). Not surprisingly, during the same period, ie, 24 hours, they did not proliferate to the same extent as sham-incubated controls, which had multiplied 2.6-fold. Normal rates of proliferation resumed soon thereafter (not shown).

On the other hand, when Ca_f was elevated from 100 μmol/L to 150 μmol/L or more, cell viability decreased sharply despite glycine (Figure 1a). The lethal consequences of ≥150 μmol/L Ca_f could not be explained by plasma membrane damage. Cells with 150 μmol/L Ca_f

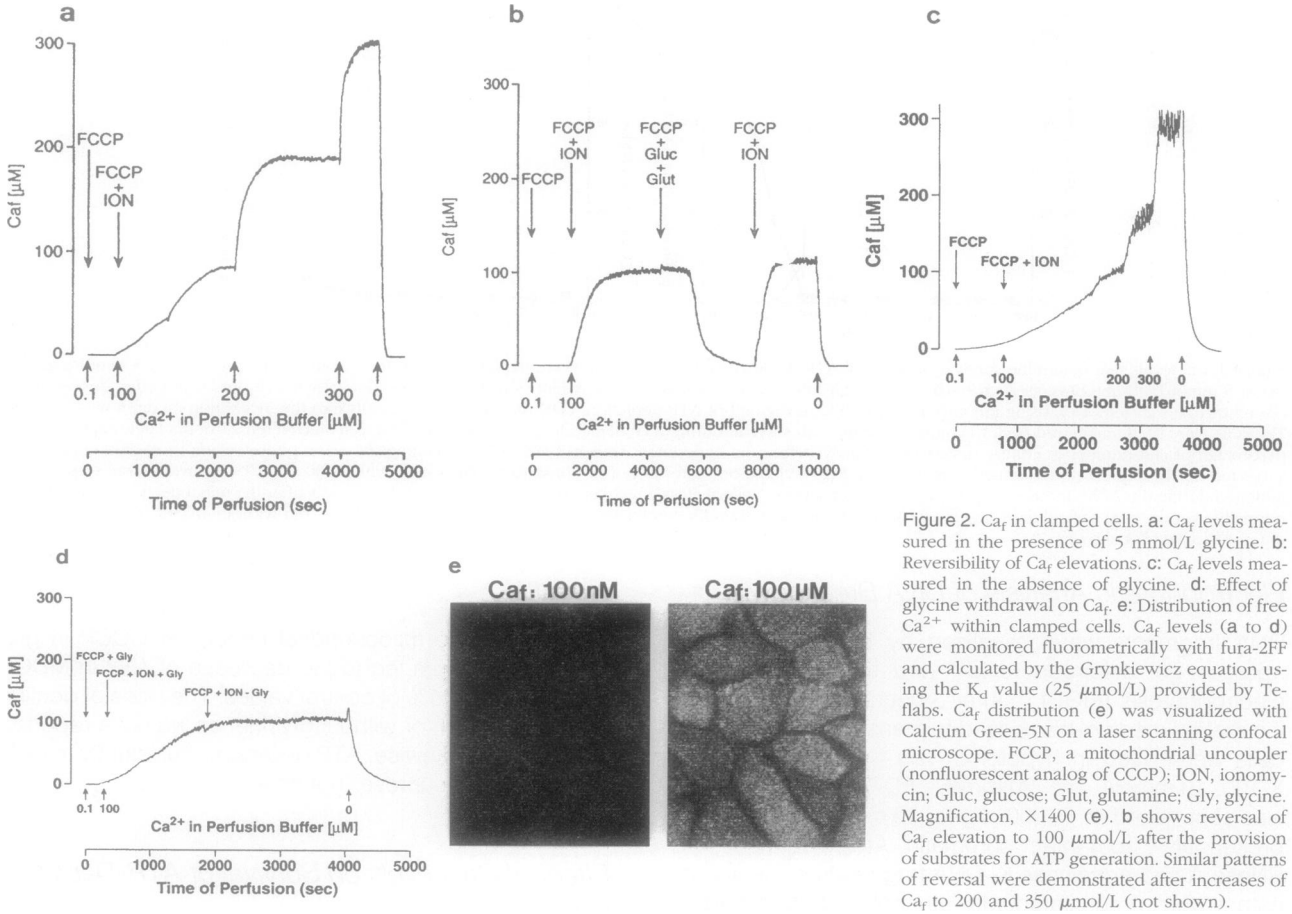

Figure 2. Ca_f in clamped cells. a: Ca_f levels measured in the presence of 5 mmol/L glycine. b: Reversibility of Ca_f elevations. c: Ca_f levels measured in the absence of glycine. d: Effect of glycine withdrawal on Ca_f. e: Distribution of free Ca^{2+} within clamped cells. Ca_f levels (a to d) were monitored fluorometrically with fura-2FF and calculated by the Grynkiewicz equation using the K_d value (25 µmol/L) provided by Teflabs. Ca_f distribution (e) was visualized with Calcium Green-5N on a laser scanning confocal microscope. FCCP, a mitochondrial uncoupler (nonfluorescent analog of CCCP); ION, ionomycin; Gluc, glucose; Glut, glutamine; Gly, glycine. Magnification, ×1400 (e). b shows reversal of Ca_f elevation to 100 µmol/L after the provision of substrates for ATP generation. Similar patterns of reversal were demonstrated after increases of Ca_f to 200 and 350 µmol/L (not shown).

lost viability, although LDH release from these cells (4.6%) did not differ from controls (2.3%) or ATP-depleted cells with 100 µmol/L Ca_f (3.0%). Cells exposed to >150 µmol/L Ca^{2+} showed severe swelling (not shown) and leaked LDH, despite the presence of glycine (Figure 1a). The additional presence in the medium of sucrose, a membrane-impermeant osmotic agent, suppressed both swelling (not shown) and LDH release (Figure 1b). However, Ca_f levels, and not the presence or absence of sucrose, determined cell viability (Figure 1b).

Ca_f in Clamped Cells

Because of the unexpected tolerance of cells to high concentrations of Ca_f, ie, ≤100 µmol/L (Figure 1), we validated the technique used to clamp Ca_f. For this purpose, four series of experiments were performed.

First, we measured Ca_f in glycine-protected ATP-depleted cells directly, using the low-affinity Ca^{2+} indicators furaptra and fura-2FF. To calculate Ca_f, we used K_d values (50 µmol/L for furaptra and 25 µmol/L for fura-2FF) provided by the manufacturer. As shown in Figure 2a, Ca_f of ATP-depleted cells increased immediately after exposure to 100 µmol/L Ca^{2+} in the presence of ionomycin. Within 20 minutes, Ca^{2+} within and outside cells reached an equilibrium (Ca_f = 94 ± 7 µmol/L; n = 5; Figure 2a). Ca_f could then be readily manipulated by changing extracellular Ca^{2+}, and new equilibria were rapidly obtained (Figure 2a). The elevations of Ca_f were reversible. As shown in Figure 2b, provision of substrates for ATP generation, ie, glucose and glutamine, resulted in a decrease of Ca_f from 100 µmol/L toward basal levels (66 nmol/L, measured with fura-2 in separate experiments) within 1 hour. Similar decreases of Ca_f from peak levels of 200 and 350 µmol/L were also demonstrated (not shown), implying the resumption of integrated energy-dependent functions in these cells also. However, these cells did not survive (Figure 1) despite the correction of Ca^{2+} homeostasis.

Second, we verified the assumption that treatment with ionomycin results in the equilibration of free Ca^{2+} across cellular membranes[32–35,40–43] by measuring the K_d of Ca^{2+}-fura-2FF complexes within ATP-depleted cells permeabilized with ionomycin. To accomplish this, we used described methods.[32–35] The free intracellular Ca^{2+} concentrations used to calculate K_d values by the Grynkiewicz equation were assumed to be those present outside, in the extracellular space. These experiments yielded K_d values (25.4 ± 3.4 µmol/L, n = 11, in the presence of glycine and 26.1 ± 5.4 µmol/L, n = 8, in the absence of glycine) that were very similar to those obtained by the manufacturer using cell-free systems and by others using the *in situ* method. The close agreement of the K_d values axiomatically indicates that the extracel-

lular Ca^{2+} concentrations used for the calculations had indeed been reached inside the cells under our experimental conditions.

Third, we excluded the possibility that glycine had either affected the equilibration of Ca^{2+} across cellular membranes or distorted the measurement itself. When the same experiment as described in Figure 2a was performed on ATP-depleted MDCK cells in the absence of glycine, the measured Ca_f levels in the presence of ionomycin and various extracellular Ca^{2+} concentrations were similar to those seen with glycine (Figure 2c). Furthermore, withdrawal of glycine from ionomycin-permeabilized cells did not result in perturbations of the measured Ca_f (Figure 2d). In this experiment, we first allowed Ca_f to increase and reach a plateau (100 μmol/L) in the presence of glycine and then washed out the amino acid by perfusion with glycine-free buffer. The measured value of Ca_f did not change over at least 30 minutes, a period sufficient for virtually all intracellular glycine to be lost by diffusion (not shown).

Fourth, intracellular free Ca^{2+} was imaged using the indicator Calcium Green-5N ($K_d \approx 3.3$ μmol/L; Molecular Probes). When Ca_f was clamped at 100 μmol/L, free Ca^{2+} was visualized to be increased in nuclei as well as the cytoplasm, assessed by confocal microscopy of 0.72-μm optical sections of cells; similarly incubated cells with Ca_f clamped at 100 nmol/L showed little signal (Figure 2e). Sections of this thickness are sufficient to resolve nuclei but not other organelles.

These studies have provided a reliable basis for the use of ionomycin to clamp intracellular Ca^{2+} at desired levels in ATP-depleted cells and examination of the cellular effects of specific concentrations of calcium ions.

Nuclear Alterations Caused by Ca_f Elevation from 100 μmol/L to 150 μmol/L

To assess whether the deleterious actions of $\geq$150 μmol/L Ca_f (Figure 1) were accompanied by structural alterations, we examined the morphology of cells exposed to different concentrations of Ca^{2+}. Shown in Figure 3a is a normal ATP-replete MDCK cell. After 3 hours of ATP depletion in the absence of glycine, cells became swollen and disrupted, with empty cytoplasm consistent with the loss of plasma membrane integrity, regardless of ambient Ca^{2+} (shown in Figure 3b for cells exposed to 100 μmol/L Ca_f). Provision of glycine during ATP depletion prevented damage to plasma membranes (Figure 3, c and d). Under these conditions, survival of cells with $\leq$100 μmol/L Ca_f and death of cells with $\geq$150 μmol/L Ca_f (Figure 1) corresponded to striking differences in nuclear morphology. As shown in Figure 3, c and d, elevation of Ca_f from 100 μmol/L to 150 μmol/L resulted in remarkable condensation of nuclear chromatin. Transition of Ca_f from $\leq$100 μmol/L to $\geq$150 μmol/L was associated with similar modifications of nuclear morphology in other groups of cells also, with or without sucrose (not shown).

To investigate the molecular basis of nuclear alterations caused by $\geq$150 μmol/L Ca_f, we analyzed DNA extracted from cells after experimental incubation. As shown in Figure 4a, elevation of Ca_f from 100 nmol/L to 10 μmol/L or 100 μmol/L did not induce DNA strand breaks. Thus, these groups of cells displayed comparable amounts of residual double-stranded DNA after alkali exposure, relative to controls. However, an abrupt increase of strand breaks in genomic DNA was triggered when Ca_f was increased to 150 μmol/L. DNA damage induced by $\geq$150 μmol/L Ca_f was further confirmed by electrophoretic analysis. Cells exposed to 150 μmol/L Ca^{2+} or more, but not those in 100 μmol/L Ca^{2+} or less, showed DNA breakdown releasing large fragments (Figure 4b). The sizes of the predominant species of released DNA fragments were between 48 and 144 kb, as shown by FIGE (Figure 4c). Breakdown of DNA into large fragments was observed only when Ca_f was $\geq$150 μmol/L, in cells with plasma membrane integrity preserved by glycine and sucrose (Figure 4, b and c). On the other hand, the omission of glycine or of sucrose in the presence of >150 μmol/L Ca^{2+} resulted in the loss of plasma membrane integrity indicated by LDH release. This was accompanied by internucleosomal breakdown of DNA, visualized as ladders (Figure 4d). However, this type of DNA damage occurred as a consequence of the loss of plasma membrane integrity, regardless of ambient Ca^{2+} concentrations (Figure 4d).[44]

Breakdown of nuclear scaffold proteins has been shown to be required for the chromatin condensation and fragmentation during apoptotic cell death and may be associated with DNA damage.[45,46] In view of the dramatic nuclear alterations triggered by $\geq$150 μmol/L Ca_f (Figures 3 and 4), we examined whether these changes were accompanied by hydrolysis of lamins, major components of the nuclear skeleton. The results are illustrated in Figure 5. Immunoblot analysis of whole-cell lysates revealed decreased amounts of intact lamins A and C in glycine-protected cells with Ca_f levels of 150 μmol/L or more. However, unlike apoptosis,[45,46] immunoblot analysis of lamin degradation in ATP-depleted cells with $\geq$150 μmol/L Ca_f did not reveal the formation of the characteristic fragments indicative of the action of caspases (Figure 5).

Discussion

The objective of this study was to determine the intracellular Ca^{2+} thresholds for triggering lethal cell damage during ATP depletion and to explore the mechanisms of Ca^{2+} cytotoxicity. Quantitative examination of these questions has not been possible before, owing to the lack of recognition of a major mechanism of damage, which is calcium independent but is completely inhibitable by glycine, a cellular constituent normally present in high concentrations. Thus, by selectively avoiding the confusing overlay of a calcium-independent injury process, the use of glycine as an experimental intervention has enabled us to ask specific questions and obtain definitive answers for the first time regarding the role played by Ca^{2+} in cell death during ATP depletion.

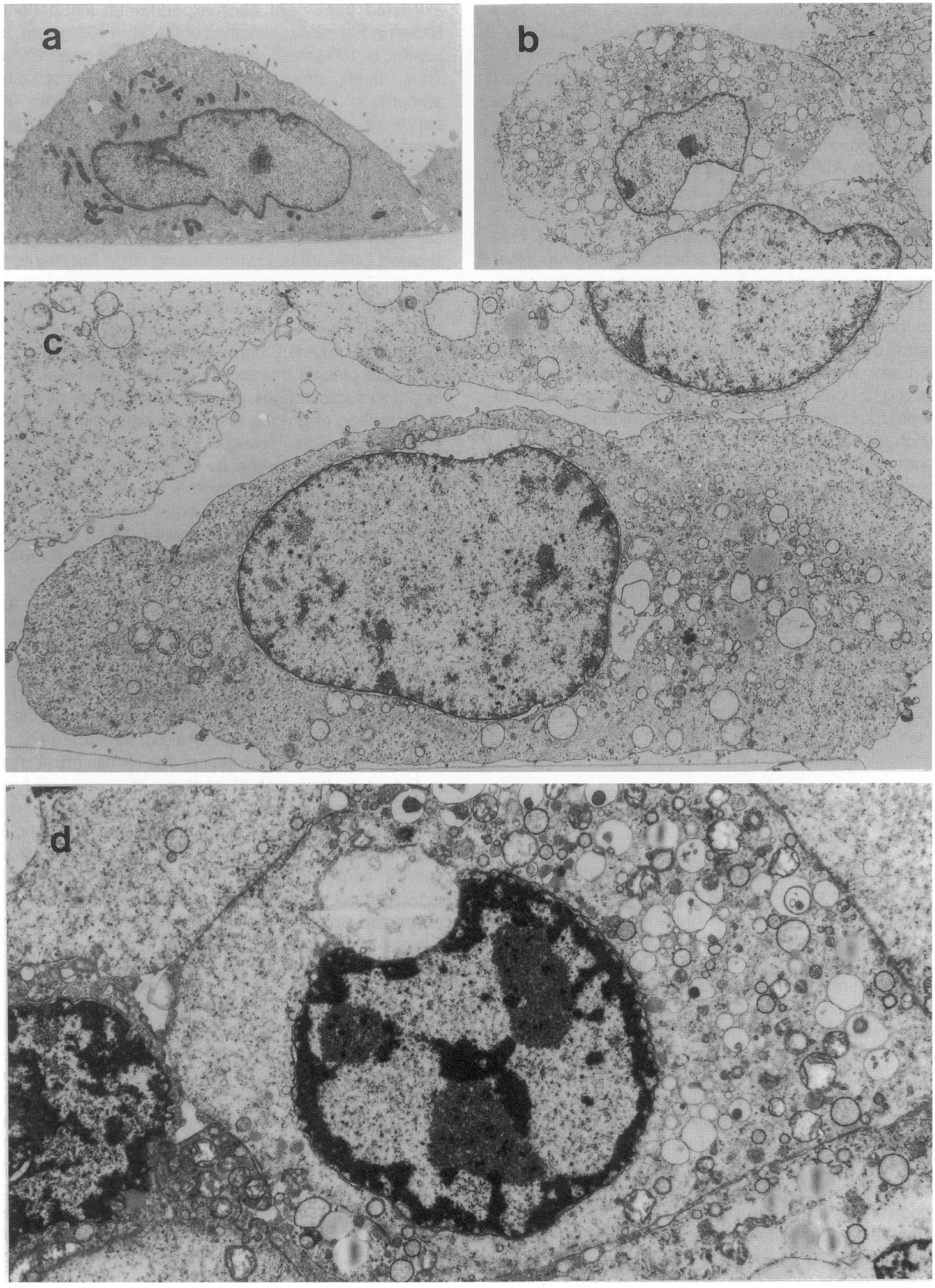

Figure 3. Morphological alterations induced by Ca_f elevation from 100 μmol/L to 150 μmol/L. Cells were incubated in KRB (**a**; control) or depleted of ATP in the absence (**b**) or presence (**c** and **d**) of glycine. Ca_f in ATP-depleted cells was clamped as described in Figure 1. Without glycine, ATP-depleted cells became necrotic regardless of Ca_f levels (shown in **b**, for cells with 100 μmol/L Ca_f). In the presence of glycine, plasma membrane integrity was preserved. Under these conditions, cells with 150 μmol/L Ca_f (**d**) but not those with 100 μmol/L Ca_f (**c**) exhibit marked condensation of chromatin. LDH release (percentage of total) was 2, 94, 4, and 14 for **a**, **b**, **c**, and **d**, respectively. Magnification, ×3200 (**a** and **b**) and ×7000 (**c** and **d**).

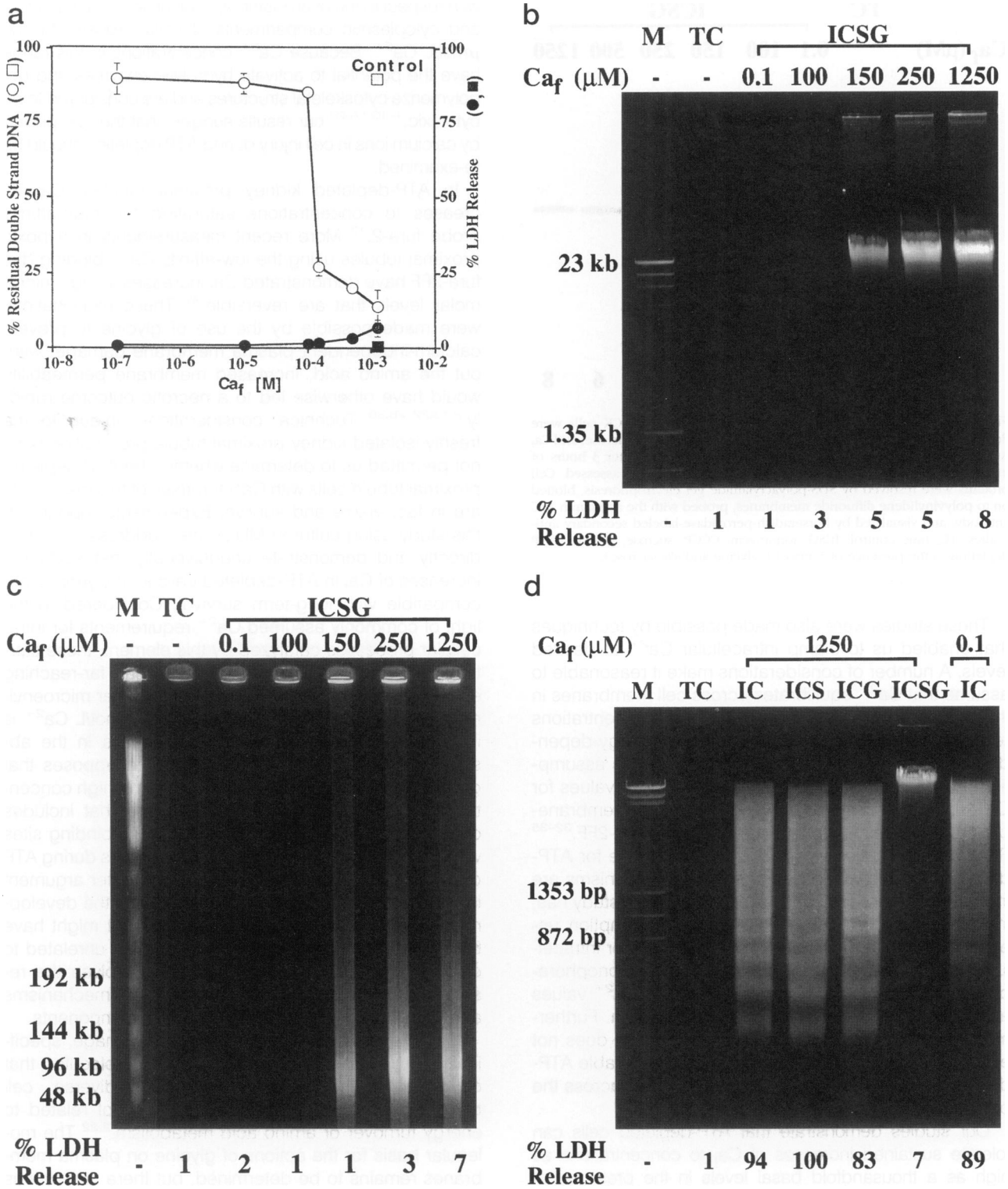

Figure 4. DNA damage induced by Ca_f exceeding 100 μmol/L. **a:** Formation of DNA strand breaks quantitated by DNA unwinding assay (mean ± SE; n = 4). **b:** DNA breakdown shown by conventional agarose gel electrophoresis. **c:** DNA breakdown shown by FIGE. **d:** Internucleosomal DNA cleavage associated with the loss of plasma membrane integrity. For **a:** to **c,** MDCK cells were depleted of ATP in the presence of 5 mmol/L glycine and 4% sucrose, and Ca_f was clamped at desired levels as described in Figure 1. For **d,** MDCK cells were depleted of ATP in the presence or absence of 5 mmol/L glycine or 4% sucrose, and Ca_f was adjusted to 1.25 mmol/L or 100 nmol/L. At the end of ATP depletion, LDH released from the cells into the incubation medium was assessed. M, molecular weight marker (λ ladders); TC, time control; ICSG, ionomycin, CCCP, sucrose, glycine; IC, ionomycin, CCCP; ICS, ionomycin, CCCP, sucrose; ICG, ionomycin, CCCP, glycine.

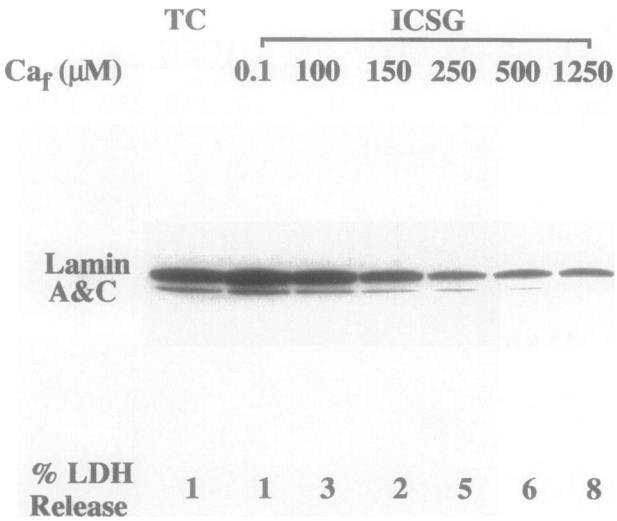

Figure 5. Lamin degradation induced by Ca_f increases. MDCK cells were depleted of ATP in the presence of 5 mmol/L glycine and 4% sucrose, and Ca_f was clamped at desired levels as described in Figure 1. After 3 hours of incubation, LDH released from cells into the medium was assessed. Cell proteins were resolved by SDS-polyacrylamide gel electrophoresis, blotted on to polyvinylidene difluoride membranes, probed with the anti-lamin A/C antibody, and visualized by horseradish-peroxidase-labeled secondary antibodies. TC, time control; ICSG, ionomycin, CCCP, sucrose, glycine (ATP depletion in the presence of 5 mmol/L glycine and 4% sucrose).

These studies were also made possible by techniques that enabled us to clamp intracellular Ca^{2+} at desired levels. A number of considerations make it reasonable to assume that Ca^{2+} equilibrates across cell membranes in the presence of Ca^{2+} ionophores if the concentrations reached are sufficient to overwhelm the energy-dependent homeostatic mechanisms.[32–35,40–43] This assumption has permitted the *in situ* calculation of K_d values for Ca^{2+}-indicator complexes within cells and membrane-bound structures for fura-2, furaptra, and fura-2FF.[32–35] Such an assumption would be particularly true for ATP-depleted cells in which Ca^{2+} pumping mechanisms are inoperative due to lack of energy. The present study has, in fact, established the validity of this assumption unequivocally by demonstrating that K_d values for intracellular Ca^{2+}-fura-2FF complexes calculated in ionophore-permeabilized cells using extracellular free Ca^{2+} values are identical to those obtained in free solution. Furthermore, the membrane-protective agent glycine does not either interfere with the Ca_f measurement or enable ATP-depleted cells to maintain a gradient of Ca^{2+} across the plasma membrane (Figure 2).

Our studies demonstrate that ATP-depleted cells can tolerate sustained increases of Ca_f to concentrations as high as a thousandfold basal levels in the presence of glycine and subsequently survive. In the experiments reported here, the extent of ATP depletion was severe, and the continuous presence of an uncoupler assured the dissipation of mitochondrial potential, preventing the sequestration of Ca^{2+} by these organelles. Therefore, we expected intracellular Ca^{2+} to not only equilibrate at extracellular levels but also to be present in equal concentrations within all cellular structures and components, ie, in cytoplasm, nuclei, and organelles. Figure 2e, in fact, confirms this inference with respect to the even distribution of the ions in the nuclear and cytoplasmic compartments of cells exposed to 100 μmol/L Ca^{2+}. Because Ca^{2+} concentrations in this range have the potential to activate hydrolytic enzymes and depolymerize cytoskeletal structures and are considered to be cytotoxic,[1–10,17–20] our results suggest that the role played by calcium ions in cell injury during ATP depletion should be re-examined.

In ATP-depleted kidney proximal tubules, Ca_f increases to concentrations saturating the high-affinity probe fura-2.[13] More recent measurements in hypoxic proximal tubules using the low-affinity Ca^{2+}-binding dye fura-2FF have demonstrated Ca_f increases to high micromolar levels that are reversible.[47] These examinations were made possible by the use of glycine to prevent calcium-independent plasma membrane damage; without the amino acid, increased membrane permeability would have otherwise led to a necrotic outcome rapidly.[2,13,22–28,30] Technical considerations unique to the freshly isolated kidney proximal tubule preparation have not permitted us to determine whether the ATP-depleted proximal tubule cells with Ca_f increases of this magnitude are in fact viable and survive. Experiments reported in this study using cultured MDCK cells address this issue directly and demonstrate unequivocally that sustained increases of Ca_f in ATP-depleted cells to 100 μmol/L are compatible with long-term survival. Considered in the light of commonly assumed Ca^{2+} requirements for intracellular processes catalyzed by this element, these startling results imply either that glycine has far-reaching effects on structure that shield critical cellular microenvironments from calcium ions or that 100 μmol/L Ca^{2+} is ineffective in activating cytotoxic pathways in the absence of ATP. The former argument presupposes that glycine, a normal intracellular constituent in high concentrations, has a ubiquitous structural role that includes control of whether Ca^{2+} has access to its binding sites where it might catalyze destructive processes during ATP depletion. We believe, however, that the latter argument is correct and that glycine merely prevents the development of defects in plasma membranes that might have been lethally injured otherwise in a manner unrelated to calcium; the recovery process simply involves the resumption of energy-dependent homeostatic mechanisms and repair of nonlethally injured cellular components.

Glycine prevents plasma membrane damage, specifically the increased permeability to macromolecules, that develops during ATP depletion in diverse cell types.[2,13,15,22–28,30,47] These effects are not related to energy turnover or amino acid metabolism.[2,22] The molecular basis for the actions of glycine on plasma membranes remains to be determined, but there are curious analogies between structure-activity relationships of protection against cell injury by cytoprotective amino acids and the biological effects of the same amino acids on inhibitory chloride channel glycine receptors in the central nervous system.[48] Based on this, and other pharmacological observations, we have proposed that the glycine-sensitive abnormality may be the development of a porous defect in a multimeric ion channel of the plasma membrane.[49] If correct, these arguments imply that the

ATP-depleted cytoplasm is remarkably resistant to calcium cytotoxicity if plasma membrane integrity is maintained and loss of cytosolic soluble contents is thereby prevented.

On the other hand, Ca_f concentrations greater than 100 μmol/L did overcome the actions of glycine and damaged the cells permanently (Figure 1). Cell death triggered by ≥150 μmol/L Ca_f was not related to loss of plasma membrane integrity, because inclusion of sucrose along with glycine allowed the membrane-protective effects of the amino acid to be fully expressed but did not promote cell survival (Figure 1). Moreover, when ATP-depleted cells with Ca_f concentrations of 100 μmol/L or more were provided with glutamine and glucose to enable ATP generation, they were able to decrease grossly elevated intracellular Ca^{2+} concentrations toward control levels (Figure 2b). These findings indicate that cells with ≥150 μmol/L Ca_f were able to mount a complex and integrated energy-dependent membrane function just as well as those with ≤100 μmol/L Ca_f. However, these cells could not survive. The loss of viability of cells with ≥150 μmol/L Ca_f is most readily explained by severe damage in nuclei reflected by chromatin condensation, lamin breakdown, and hydrolysis of DNA. Thus, our results not only have documented the unexpectedly high Ca^{2+} levels required to trigger cell death during ATP depletion but also have provided novel insights into mechanisms of Ca^{2+} cytotoxicity.

Finally, the high tolerance of ATP-depleted cells to Ca_f elevations needs to be considered in the context of Ca^{2+} toxicity that is known to be expressed in the presence of ATP. Pharmacologically induced Ca^{2+} overload as well as pathological fluxes of Ca^{2+} into cells through damaged plasma membranes can result in excessive uptake of the ions into mitochondria and damage the organelles.[19,20] However, this type of injury is dependent on the ability of cells to generate energy and mount mitochondrial potential, conditions that are not obtained during severe ATP depletion. Whether cells will recover after injury due to ATP depletion *in vivo* caused by conditions such as ischemia may indeed hinge on their ability to maintain ion homeostasis as energy levels increase and thus avoid Ca^{2+}-mediated damage. However, this is likely to be determined by the state of preservation of cell structure and, in view of our previous and present findings, by the availability of glycine.

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