

Cell Calcium, Cell Injury and Cell Death

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The role of calcium in cell injury has been the subject of much recent investigation. The movement and redistribution of this cation from extra to intracellular compartments and the calcium shifts between intracellular compartments may well play a determinate role in the cell's reaction to injury. Therefore, data of such shifts and their correlation with morphological, biochemical and cytoskeletal studies will provide a better understanding of these processes. To study the effects of calcium regulation on acute lethal anoxic injury and the effects of inhibition of respiration with cyanide, three experimental systems were utilized: Ehrlich ascites tumor cells, isolated rabbit proximal tubule segments and suspended or cultured rat proximal tubule cells. Although our data showed no correlation between total cell calcium and cell death except in highly selected cell systems, they did indicate that calcium can be an important control variable. Therefore, massive increases in total cell calcium, as seen in $\text{Ca}_3(\text{PO}_4)_2$ precipitation in mitochondria, must be a secondary event and represent the modern day equivalent of the classical dystrophic calcification seen by pathologists in the past. Although the involvement of extracellular calcium in cell death may well be significant in some cell types, redistribution of calcium within the intracellular compartments may play an even more important role.

Introduction

For the past 20 years we have been characterizing the relationship between cellular ion regulation, cell injury and cell death. We noted that many characteristics and kinetics of acute lethal injury in rat renal slices (1), toad bladders (2), and isolated flounder renal tubules (3,4) could be reproduced by agents or conditions that interfere with ion permeability and/or transport at the plasma membrane. At the same time, it was also observed that there was some correlation between cellular Ca content and cell death. For example, when the effects of HgCl_2 on the rat kidney were studied, results showed that mitochondrial Ca increased markedly from zero time to 48 hr as the cells became necrotic, while Na increased earlier up to 12 hr and then leveled off (5). Analogous calcification occurs with CCl_4 in hepatocytes. Although this relationship between Ca content and cell death, often referred to as "dystrophic" calcification, has been noted by pathologists for many years, what remains unclear is whether it is merely a secondary effect or whether Ca deregulation acts in killing the cell. On the other hand, in many systems, extracellular Ca is essential for life, especially in non-neoplastic cells.

Here, we have focused on the effects of Ca regulation

on acute lethal anoxic injury and the effects of inhibition of respiration with cyanide. Three experimental systems were utilized: Ehrlich ascites tumor cells (EATC), isolated rabbit proximal tubule segments, and cultured or suspended purified rat proximal tubular epithelial cells.

Results

In the first experimental system, EATC were incubated in Krebs-Ringer phosphate (KRP) buffer, gassed with purified nitrogen (6). Viability was assessed with nigrosin (7-9). Figure 1 shows the effects on K and ATP and also indicates the "point of no return." In Figure 2, ATP is plotted against cell viability. This curve exhibits a threshold and a region of insensitivity to anoxia, thus illustrating that ATP as a single control variable is not revealing. In order to further examine our hypothesis, we pooled a variety of injuries in EATC and plotted Ca versus cell viability (Fig. 3). This curve illustrates that no correlation exists, but if each experiment is plotted separately, there is a clear trend.

Some reflection of this can be seen in Figure 4, which compares Ca content and viability in aerated and anoxic EATC treated with the Ca ionophore A23187. Note that in the aerated cells total cell Ca increases markedly with little effect on viability, whereas with anoxia the effects are synergistic and rapidly lead to death. We interpret this to mean that intracellular buffer systems

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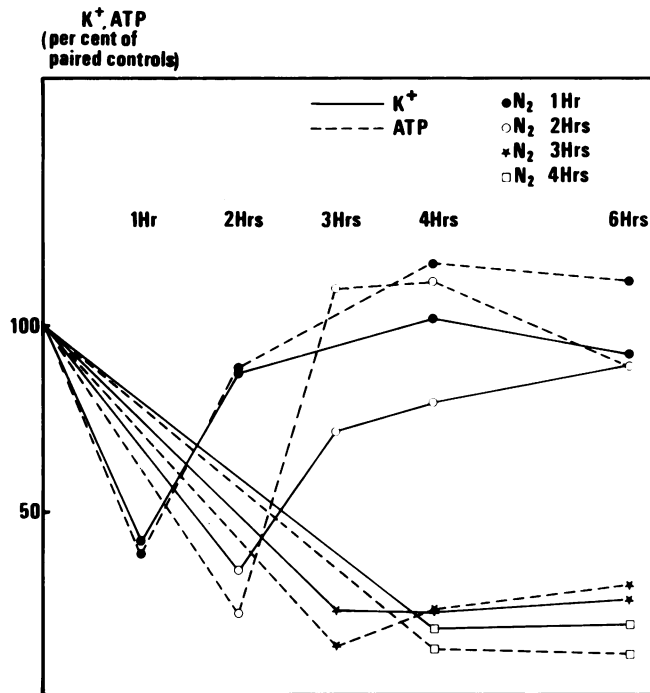


FIGURE 1. Plot of K^+ and ATP levels vs. time in anoxic Ehrlich ascites tumor cells. At 1, 2, 3, 4, and 6 hr the cells were aerated. Note that after 2 hr the cells did not recover K^+ or ATP levels, indicating that the point of no return was somewhere between 2 and 3 hr. Reproduced with permission from Trump and Laiho (6).

such as endoplasmic reticulum (ER) and mitochondria can readily compensate for increased cytosol Ca if an energy source is present. Thus, cellular ATP and Ca appear to be cooperative control variables in this system.

In another series of experiments, viability of EATC was tabulated as a function of time in media where ionized Ca was modified. By 1 hr, anoxia in the absence of added Ca or in the presence of EDTA resulted in slight but significant increases in cell viability, although by 3 hr, there was relatively little effect; i.e., reducing extracellular ionized Ca did delay but did not prevent cell death. We also observed that in the presence of A23187, cell killing was even more rapid. In contrast to this, however, anoxia in the presence of EDTA with or without ionophore or anoxia with no Ca added and in the presence of ionophore significantly increased viability by 3 hr.

We had previously noted that reduction of extracellular pH delayed cell killing following anoxia or other injuries whereas slight increases in pH enhanced it (10-14). This could relate to competitive effects of protons and Ca on effector systems such as calmodulin. Such antagonistic effects have been noted in other systems. Also, Ca-proton exchange mechanisms at cell membranes have been suggested.

The effects on morphology are shown in Figures 5 and 6. Normally, EATC are spherical in shape with well-developed surface microvilli. Very quickly after anoxia,

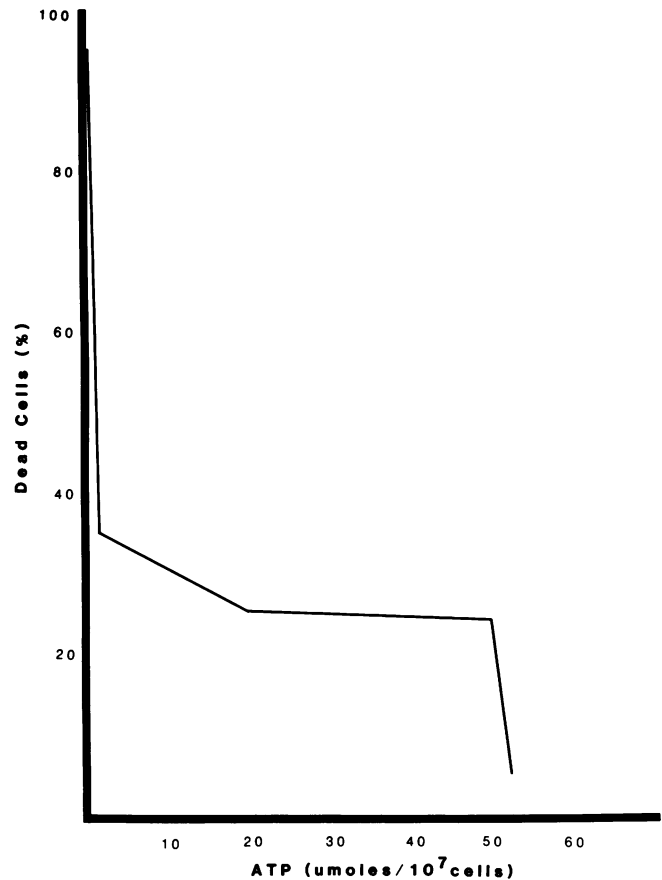


FIGURE 2. Plot of cell viability vs. ATP in a variety of cell injuries in Ehrlich ascites tumor cells (4-6).

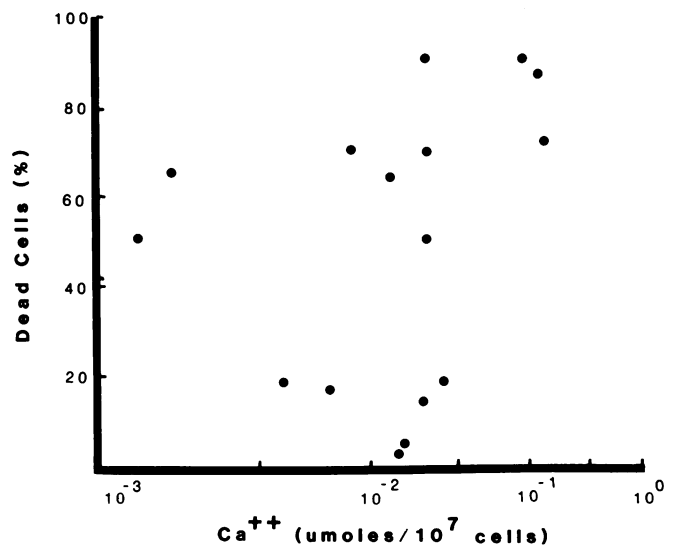


FIGURE 3. Plots of Ca content vs. dead cells from a series of experiments in Ehrlich ascites tumor cells (4-6).

cell shape changes markedly and blebs occur at the cell surface as observed by scanning electron microscopy (SEM). Moreover, these blebs can often be observed to detach and float away. Such changes always begin

during the reversible phase and sometimes within seconds as observed with the addition of A23187. Using transmission electron microscopy (TEM), the cells at this time show condensed mitochondria, dilated ER and clumping of nuclear chromatin. At later intervals when

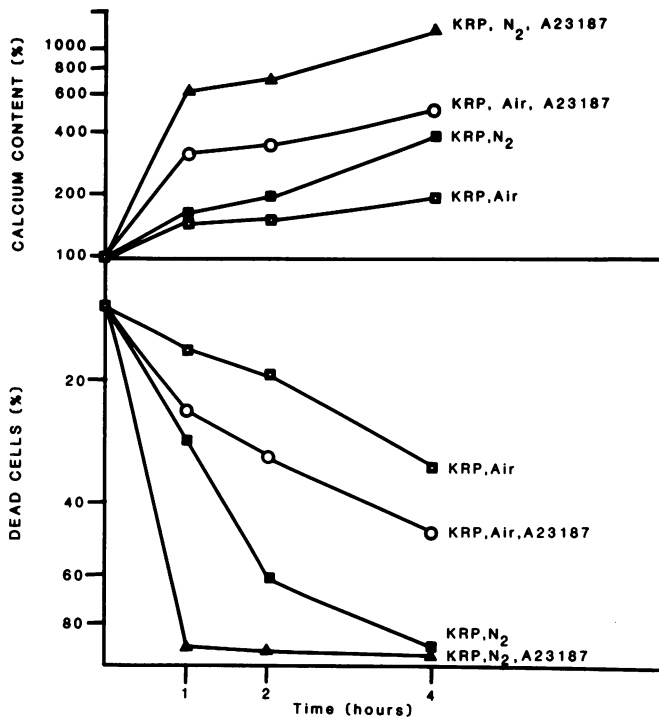


FIGURE 4. Plots of Ca content and dead cells vs. time in control and anoxic Ehrlich ascites tumor cells with and without addition of Ca ionophore A23187.

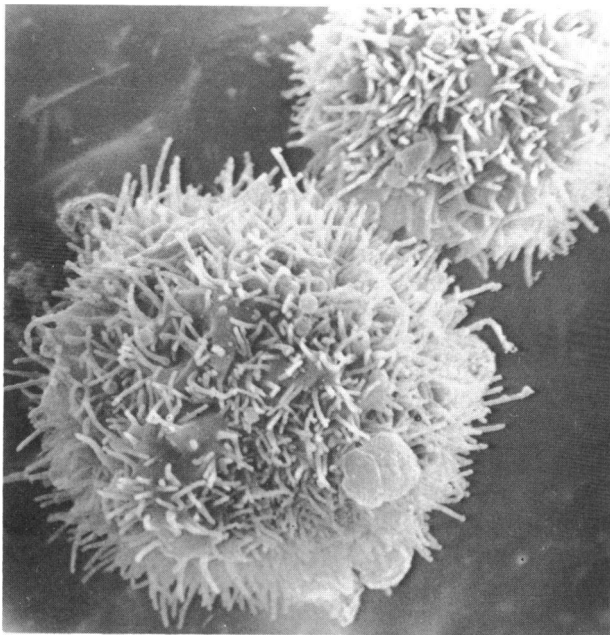


FIGURE 5. Scanning electron micrograph of control (untreated) Ehrlich ascites tumor cells. Reproduced with permission from Trump et al. (18).

the cells have passed the "point of no return," the cell surfaces tend to become smooth with irregular pitting. TEM reveals swollen mitochondria with flocculent densities, fragmentation of ER and interruptions of the integrity of the plasma membrane. No mitochondrial calcifications were seen.

Other experiments were performed on cells in aerated KRP with the addition of A23187. Again, rapid cell shape changes occurred with large blebs forming at the cell surface as noted by SEM. By TEM, the cells revealed increased numbers of autophagosomes and numerous condensed mitochondria. It is to be emphasized that under these conditions the cells showing such shape changes were, in general, perfectly viable. In conjunction with the increased intracellular Ca content and the obvious shape changes, cytoskeletal modifications undoubtedly also occur. It is well known that high concentrations of Ca depolymerize microtubules and inhibit actin polymerization; however, the specific nature of the cytoskeletal changes and the resulting effect on cell activities remains to be determined.

Rabbit proximal tubule segments were isolated by a modification of the method of Balaban et al. (15). The tubule suspensions were preincubated in minimum essential medium (MEM) or Ca-free MEM for 10 min prior to the beginning of each experiment. In the case of the experiments with zero Ca, the solution contained 0.15 mM EGTA. Viability was measured by LDH release.

When rabbit proximal tubules were incubated in 10^{-3} KCN in the presence or absence of Ca, progressive cell killing occurred over a 2-hr period. In Figure 7, the

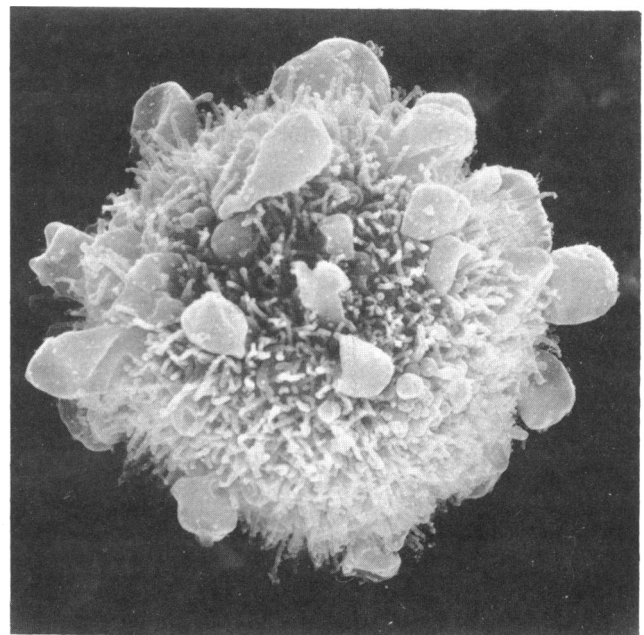


FIGURE 6. Scanning electron micrograph of Ehrlich ascites tumor cells 60 min after continual gassing with nitrogen (anoxia). Reproduced with permission from Trump et al. (18).

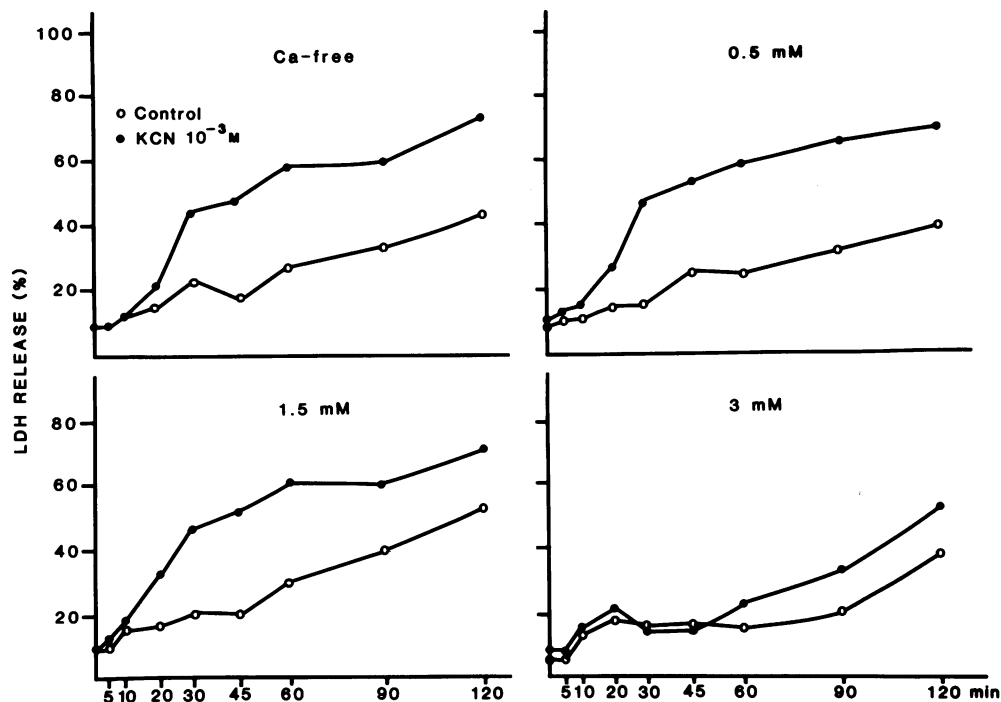


FIGURE 7. Plot of viability (measured by LDH release) vs. time in isolated rabbit kidney proximal tubules incubated with and without KCN at varying concentrations of extracellular Ca.

killing curves are compared in a Ca-free medium and in media with 0.5, 1.5 and 3.0 mM Ca. Notice that no difference occurred except in the 3 mM medium where significant reduction in the amount of cell killing occurred. Examination of thin sections by TEM showed progressive changes beginning, as with the EATC, with condensation of mitochondria, dilatation of ER, swelling of the cytosol and clumping of nuclear chromatin. At later stages, as the cells began to die, to this was added mitochondrial swelling with flocculent densities and fragmentation of plasmalemma and all intracellular membrane systems.

Rat kidney proximal tubule cells were isolated by the method of Sato et al. (16,17) using collagenase perfusion with or without Ca, depending on the experiment. All cells were preincubated in MEM with or without Ca for 20 min prior to the initiation of experiments. Viability was measured by LDH release.

When isolated rat proximal tubule cells were incubated anoxically with or without Ca, there was very little difference; if anything, cell killing was more prominent in the Ca-free medium. Morphologically, early blebbing occurred during the reversible phase in most cells as seen by SEM. Similar blebbing was seen by TEM in addition to deep invaginations associated with microfilaments. Indirect immunofluorescence staining of cells grown on coverslips revealed striking changes in the arrangement and population of actin filaments and differences in cell shape and size. Later changes revealed increased actin staining in circular areas representing either cytoplasmic vacuoles or cellular blebs.

Discussion

It is our hypothesis that increased ionized Ca and/or Ca-calmodulin complexes in the cytosol are an important factor in the initiation of cell death following a variety of cell injuries (18-21). Increases in cytosol Ca can result from extracellular entry and/or redistribution from intracellular compartments, e.g., the mitochondria and ER. In EATC subjected to anoxia, reduction of extracellular Ca retards cell death; however, more of an effect is seen if EDTA or A23187 is present. We hypothesize that this is because the intracellular buffer systems are inactivated in the absence of ATP. Therefore, there is a minimal effect of retarding entry since efflux from mitochondria and ER also presumably occurs insuring that the amount of Ca available to raise the level of ionized Ca is not limiting. Certainly, marked increases in intracellular Ca were produced in our experiments with the EATC system in which A23187 was added to cells with presumably intact intracellular buffers.

The situation can be markedly different when the primary injury involves direct attack on cell membrane systems, for example, carbon tetrachloride-induced lipid peroxidation or activation of complement. In these cases, damage occurs at the cell membrane while the production of ATP by mitochondria and mitochondrial membrane integrity are initially unaffected or only slightly affected. Cytosol Ca levels rise to the point where mitochondria begin sequestering Ca. Even in the presence of ATP, the cell membrane extrusion system is

$\text{Ca}_3(\text{PO}_4)_2$ and ultimately is in the form of Ca hydroxyapatite.

Our studies also show that no correlation exists between total cell Ca and cell death if a variety of experimental conditions are explored; yet in each



FIGURE 8. Flow chart depicting our current working hypothesis on the interactions of Ca, cell injury and cell death. Reproduced with permission from Trump and Berezesky (26).

condition, Ca increases as cell killing proceeds. It appears that $\text{Ca}_3(\text{PO}_4)_2$ precipitation within the mitochondria, which leads to massive increases in total cell Ca, represents a secondary event and is the modern equivalent of the classical "dystrophic" calcification noted by pathologists in the past.

One striking result in our experiments was the observation that addition of the Ca ionophore A23187 in the absence of extracellular Ca or addition of EDTA to the KRP with or without the Ca ionophore was significantly protective even after 3 hr of anoxia. This effect may be interpreted as follows. In the presence of a lipid-soluble Ca ionophore, Ca moves across membrane barriers down its concentration gradient. With Ca-free extracellular media, any Ca that is redistributed within the cell, for example, by release from mitochondria or ER, is rapidly dispersed from the cell and diluted into the medium (which is very large relative to the cell). The same is true of EDTA which is able to effectively remove Ca from its ionized state. These results, we believe, are again compatible with a role of increased ionized cytosol Ca in producing cell killing.

Finally, the difference between neoplastic and non-neoplastic cells may be of significance. Recently, in reviewing the literature on relationships between cell ion content and the cytoskeleton in normal and neoplastic cells, it was obvious that there are marked differences between normal and malignant cells, especially regarding the Ca growth requirements.

In conclusion, the relationship between cell Ca and cell death is complex; total cell Ca does not appear to predict cell death except in some highly selected cell systems. The difference between suspension and monolayer cultures as exemplified in the present study and in those of Casini and Farber (22), Smith et al. (23), and Stacey and Klaassen (24) still needs explanation. Certainly, in contrast to hepatocytes or proximal tubule cells, EATC are well adapted for life in suspension. Incubation of any non-neoplastic epithelium in a Ca-free medium represents a rather abnormal destabilizing condition; therefore, effects of Ca-entry blockers and calmodulin antagonists need to be characterized. In the experiments of Joseph et al. (25), it was noted that the buffer systems might already be Ca-loaded in freshly isolated hepatocytes. The membrane blebbing seen during the reversible phase in their system may well specifically reflect Ca effects on the cytoskeleton. Our studies on the sequential changes in actin distribution by immunofluorescence are consistent with this view.

Figure 8 illustrates our concept of the forces involved; obviously, these will differ with the precise type of cell injury. However, in many systems, including anoxia, inhibition of respiration and total ischemia, the redistribution of Ca within intracellular compartments may well be as important or more important than influx from the extracellular fluid. Ca regulation may differ in various types of cells, e.g., in excitable cells, Na-Ca exchange may be an important factor while in other

cells Ca-ATPase may play a role at the cell membrane. In addition to the plasmalemma, intracellular buffer systems include the ER and the mitochondria which, in the presence of energy, can regulate up to their capacity the level of Ca and Ca-calmodulin complexes in the cytosol. In theory, in any particular cell injury, there may be any permeation of Na or Ca entry or deregulation, e.g., a particular injury may primarily affect cell membrane permeability, ATP levels, mitochondrial buffer capacity or ER uptake. Several examples of this are shown in Figure 8. Modulations include antagonism by protons or modification of entry, e.g., with Ca entry blockers. In the lower half of Figure 8, some of the sublethal and lethal effects of Ca deregulation are shown, including diminished cell-cell communication, modulation of cytoskeleton, activation of phospholipase, and stimulation of macromolecular synthesis. Phospholipase activation appears to play an important role in the generation of lethal cell injury because first reversible and then irreversible changes are initiated. This pathway also activates the prostanoid metabolism, some of which may act as feedbacks which modify Ca entry. In the case of lethal injury, it is our view that irreversible permeability modifications in mitochondrial and cell membrane bilayers result in irreversible cell injury. Note that mitochondrial calcification in the form of calcium phosphate represents a reflection of this type of initial interaction since mitochondrial calcification only occurs with injuries that affect Ca deregulation through the cell membrane or ER without primary effects on mitochondrial function.

This study was supported by NIH Grant AM 15440. BFT is an American Cancer Society Professor of Oncology. This is contribution #1608 from the Cellular Pathobiology Laboratory.

REFERENCES

1. Trump, B. F., Strum, J. M., and Bulger, R. E. Studies on the pathogenesis of ischemic cell injury. I. Relation between ion and water shifts and cell ultrastructure in rat kidney slices during swelling at 0–4°C. *Virchows Arch. Abt. B Zellpath.* 16: 1–34 (1974).
2. Saladino, A. J., Bentley, P. J., and Trump, B. F. Ion movements in cell injury. Effect of amphotericin B on the ultrastructure and function of the epithelial cells of the toad bladder. *Am. J. Pathol.* 54: 421–466 (1969).
3. Trump, B. F., and Bulger, R. E. Studies of cellular injury in isolated flounder tubules. III. Light microscopic and functional changes due to cyanide. *Lab. Invest.* 18: 721–730 (1968).
4. Trump, B. F., and Bulger, R. E. Studies of cellular injury in isolated flounder tubules. IV. Electron microscopic observations of changes during the phase of altered homeostasis in tubules treated with cyanide. *Lab. Invest.* 18: 731–739 (1968).
5. Trump, B. F., Croker, B. P., and Mergner, W. J. The role of energy metabolism, ion and water shifts in the pathogenesis of cell injury. In: *Cell Membranes: Biological and Pathological Aspects* (G. W. Richter, D. G. Scarpelli and N. K. Kaufman, Eds.), Williams and Wilkins, Baltimore, 1971, pp. 84–128.
6. Trump, B. F., and Laiho, K. U. Studies of cellular recovery from injury. I. Recovery from anoxia in Ehrlich ascites tumor cells. *Lab. Invest.* 33: 706–711 (1975).
7. Laiho, K. U., Shelburne, J. D., and Trump, B. F. Observations on

- cell volume, ultrastructure, mitochondrial conformation and vital-dye uptake in Ehrlich ascites tumor cells: effects of inhibiting energy production and function of the plasma membrane. *Am. J. Pathol.* 65: 203-230 (1971).
8. Laiho, K. U., and Trump, B. F. Relationship of ionic, water, and cell volume changes in cellular injury of Ehrlich ascites tumor cells. *Lab. Invest.* 31: 207-215 (1974).
 9. Laiho, K. U., and Trump, B. F. The relationship between cell viability and changes in mitochondrial ultrastructure, cellular ATP, ion and water content following injury of Ehrlich ascites tumor cells. *Virchows Arch. Abt. B. Zellpath.* 15: 267-277 (1974).
 10. Penttilä, A., and Trump, B. F. Extracellular acidosis protects Ehrlich ascites tumor cells and rat renal cortex against anoxic injury. *Science* 185: 277-278 (1974).
 11. Penttilä, A., and Trump, B. F. Studies on modification of the cellular response to injury. I. Protective effect of acidosis on *p*-chloromercuribenzenesulfonic acid-induced injury of Ehrlich ascites tumor cells. *Lab. Invest.* 32: 690-695 (1975).
 12. Penttilä, A., and Trump, B. F. Studies on the modification of the cellular response to injury. II. Electron microscopic studies on the protective effect of acidosis on anoxic injury of Ehrlich ascites tumor cells. *Virchows Arch. B Cell Pathol.* 18: 1-16 (1975).
 13. Penttilä, A., and Trump, B. F. Studies on the modification of the cellular response to injury. III. Electron microscopic studies on the protective effect of acidosis on *p*-chloromercuribenzenesulfonic acid (PCMBs) induced injury of Ehrlich ascites tumor cells. *Virchows Arch. B Cell Pathol.* 18: 17-34 (1975).
 14. Penttilä, A., Glaumann, H., and Trump, B. F. Studies on the modification of the cellular response to injury. IV. Protective effect of extracellular acidosis against anoxia, thermal, and *p*-chloromercuribenzenesulfonic acid treatment of isolated rat liver cells. *Life Sci.* 18: 1419-1430 (1976).
 15. Balaban, R. R., Soltoff, S. P., Storey, J. M., and Mandel, L. J. Improved renal cortical tubule suspension. Spectrophotometric study of oxygen delivery. *Am. J. Physiol.* 238: F50-F59 (1980).
 16. Sato, T., Trifillis, A., and Trump, B. F. Oxygen uptake and adenine nucleotides of isolated proximal tubule cells in the rat kidney. *Fed. Proc.* 37: 265 (1978).
 17. Sato, T., Berezesky, I. K., Nakatani, T., and Trump, B. F. The role of extracellular (EC) calcium on isolated rat proximal tubule cells. *J. Cell Biol.* 95: 466 (1982).
 18. Trump, B. F., Berezesky, I. K., Laiho, K. U., Osornio, A. R., Mergner, W. J., and Smith, M. W. The role of calcium in cell injury. A review. *Scanning Electron Microsc.* 2: 437-462 (1980).
 19. Trump, B. F., Berezesky, I. K., and Phelps, P. C. Sodium and calcium regulation and the role of the cytoskeleton in the pathogenesis of disease. A review and hypothesis. *Scanning Electron Microsc.* 2: 434-454 (1981).
 20. Trump, B. F., Berezesky, I. K., and Phelps, P. C. The role of altered sodium and calcium regulation and the cytoskeleton in the pathogenesis of human disease. *J. Clin. Electr. Microsc.* 14: 5-6 (1981).
 21. Trump, B. F., Sato, T., Berezesky, I. K., and Laiho, K. U. Cell calcium (Ca_i) and anoxia cell death. *Fed. Proc.* 42: 661 (1983).
 22. Casini, A. F., and Farber, J. L. Dependence of the carbon tetrachloride-induced death of cultured hepatocytes on the extracellular calcium concentration. *Am. J. Pathol.* 105: 138-148 (1981).
 23. Smith, M. T., Thor, H., and Orrenius, S. Toxic injury to isolated hepatocytes is not dependent on extracellular calcium. *Science* 213: 1257-1259 (1981).
 24. Stacey, N. H., and Klaassen, C. D. Lack of protection against chemically induced injury to isolated hepatocytes by omission of calcium from the incubation medium. *J. Toxicol. Environ. Health* 9: 267-276 (1982).
 25. Joseph, S. K., Coll, K. E., Cooper, R. H., Marks, J. S., and Williamson, J. R. Mechanisms underlying calcium homeostasis in isolated hepatocytes. *J. Biol. Chem.* 258: 731-741 (1983).
 26. Trump, B. F., and Berezesky, I. K. Role of sodium and calcium regulation in toxic cell injury. In: *Drug Metabolism and Drug Toxicity* (J. R. Mitchell and M. G. Horning, Eds.), Raven Press, New York, 1984, Chapt. 13.