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C E McCoy, ... , E A Alexander, J H Schwartz

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Research Article

An elevation in cytosolic free calcium (Cai) produced by cellular ATP depletion may contribute to the initiation of cytotoxic events in renal ischemia. To evaluate whether ATP depletion results in a rise in Cai we examined the effect of cyanide and 2-deoxy-D-glucose on the Cai of Madin-Darby canine kidney cells. Exposure to the metabolic inhibitors resulted in a rise in Cai from 112 +/- 11 to 649 +/- 99 nM in 15 min. This combination of metabolic inhibitors also resulted in a decrement of cell ATP to 11 +/- 2% of control by 15 min. Experiments that were performed with other metabolic inhibitors confirm that the increment in Cai is due to inhibition of ATP synthesis. With the removal of cyanide and 2-deoxy-D-glucose, Cai recovered to 101 +/- 16 nM. In the absence of extracellular calcium activity (Ca0), Cai declined from 127 +/- 7 to 38 +/- 6 nM, whereas with cyanide plus 2-deoxy-D-glucose in the absence of Ca0 the Cai rose from 108 +/- 21 to 151 +/- 28 nM. Because the rise in Cai produced by ATP depletion in the absence of Ca0 is significantly less than that which occurs in the presence of Ca0, influx of Ca0 is necessary for the maximal rise of Cai. The rise in Cai that occurred in the absence of Ca0 suggests [...]

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Adenosine Triphosphate Depletion Induces a Rise in Cytosolic Free Calcium in Canine Renal Epithelial Cells

C. E. McCoy, A. M. Selvaggio, E. A. Alexander, and J. H. Schwartz

Thorndike Memorial Laboratory, Renal Section, Boston City Hospital and Departments of Medicine and Physiology, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract

An elevation in cytosolic free calcium (Ca_i) produced by cellular ATP depletion may contribute to the initiation of cytotoxic events in renal ischemia. To evaluate whether ATP depletion results in a rise in Ca_i we examined the effect of cyanide and 2-deoxy-D-glucose on the Ca_i of Madin-Darby canine kidney cells. Exposure to the metabolic inhibitors resulted in a rise in Ca_i from 112 ± 11 to 649 ± 99 nM in 15 min. This combination of metabolic inhibitors also resulted in a decrement of cell ATP to $11 \pm 2\%$ of control by 15 min. Experiments that were performed with other metabolic inhibitors confirm that the increment in Ca_i is due to inhibition of ATP synthesis. With the removal of cyanide and 2-deoxy-D-glucose, Ca_i recovered to 101 ± 16 nM. In the absence of extracellular calcium activity (Ca_0), Ca_i declined from 127 ± 7 to 38 ± 6 nM, whereas with cyanide plus 2-deoxy-D-glucose in the absence of Ca_0 the Ca_i rose from 108 ± 21 to 151 ± 28 nM. Because the rise in Ca_i produced by ATP depletion in the absence of Ca_0 is significantly less than that which occurs in the presence of Ca_0 , influx of Ca_0 is necessary for the maximal rise of Ca_i . The rise in Ca_i that occurred in the absence of Ca_0 suggests that the release of calcium from intracellular stores contributes to the increment in Ca_i seen with ATP depletion. TMB-8, an inhibitor of calcium release from intracellular stores, blunted the rise in Ca_i by nearly 50%. Neither verapamil nor nifedipine inhibited the rise in Ca_i . This study demonstrates that ATP depletion induced by the metabolic inhibitors cyanide and 2-deoxy-D-glucose is associated with a rapid and reversible increase in Ca_i . Both Ca_0 influx and Ca_i redistribution contribute to this rise.

Introduction

It has been proposed that a rise in cytosolic calcium may play an initiating role in the cellular events that lead to epithelial cell injury during ischemic acute renal failure (1, 2). However, experimental evidence supporting this hypothesis has been inconclusive. Because the concentration of cytosolic free calcium (Ca_i)¹ is 10,000-fold less than Ca_0 , energy in the form of

ATP is required to maintain the Ca_i . ATP hydrolysis is required to provide energy for the calcium pumps that exist in the plasma membrane and the membrane of the endoplasmic reticulum (3). These calcium pumps transport calcium ions out of the cell and into storage sites within the endoplasmic reticulum. A Na^+/Ca^{++} exchanger exists in the plasma membrane and probably participates in the regulation of Ca_i (4). Energy is provided by the transcellular sodium gradient that is maintained by the plasma membrane Na^+/K^+ ATPase. Finally, calcium is taken up by mitochondria and linked to this organelle's proton pump (5). Since cellular ATP depletion is the sine qua non of ischemic cellular injury, logic would dictate that Ca_i should rise during ischemia.

An elevated Ca_i during cell ischemia could participate in the pathogenesis of cell injury in several ways. Xanthine dehydrogenase is converted to xanthine oxidase by a calcium-dependent process (1, 2). An increased concentration of xanthine oxidase could lead to the accumulation of toxic oxygen radicals during reflow. Phospholipases can be activated by a rise in Ca_i (6). These enzymes may produce changes in cell membrane permeability and generate potentially cytotoxic fatty acids. Calcium has also been shown to accumulate in mitochondria in response to elevations in ambient calcium concentration as well as during ischemia (7-9), a response associated with defects in oxidative phosphorylation (7). Other effects of an elevated Ca_i are less well defined but may include the activation of calcium-sensitive ion channels (10) and perturbations of cytoskeletal components (2). While each of these events could play an important role in the pathogenesis of ischemic cellular injury it has yet to be established whether they do.

Does Ca_i rise during cell ischemia? The purpose of these studies was to answer this fundamental question. We chose as a model of cellular ischemia cultured renal epithelial cells treated with inhibitors of ATP synthesis. We examined the effect of ATP depletion on Ca_i and the contribution of extracellular calcium (Ca_0) influx and intracellular redistribution of sequestered calcium. It is clear from our studies that cellular ATP depletion induced by metabolic inhibitors is associated with a reversible rise in Ca_i . Ca_i and Ca_0 contribute to this rise.

Methods

Cell culture

Madin-Darby canine kidney (MDCK) cells were obtained from American Type Tissue Culture (Rockville, MD). Cells were grown in 75-cm² cell culture flasks (GIBCO, Grand Island, NY). The culture media used was DME (GIBCO) with 10% FCS (HyClone Laboratories, Inc., Logan, UT), 50 μ g/ml penicillin and 50 μ g/ml streptomycin. Cells were grown in an incubator at 37°C and aerated with 95% air and 5% CO₂. For passage, cells were rinsed with a calcium- and magnesium-free PBS solution. This was followed by exposure to 2 cm³ of 50 mg% trypsin and 20 mg% EGTA in calcium- and magnesium-free Hanks' solution (GIBCO) for 5-15 min.

Address all correspondence to Dr. John H. Schwartz, Thorndike Laboratory 416, Boston City Hospital, 818 Harrison Avenue, Boston, MA 02118.

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1. Abbreviations used in this paper: Ca_i , cytosolic free calcium; Ca_0 , extracellular free calcium; MDCK, Madin-Darby canine kidney; TMB-8, 3,4,5-trimethoxybenzoate 8-(*N,N*-diethylamino)octyl ester.

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Cells used for Ca_i measurement were passaged to 2.5 cm² tissue culture wells (Costar Corp., Cambridge, MA) containing 12 × 12-mm glass coverslips. Those cells used in ATP assays were grown in 2.5-cm² tissue culture wells without coverslips. Cells were used 48–96 h after passage and after reaching confluence.

Ca_i activity measurement

MDCK cells were incubated in culture media containing 10 μM fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) and 0.04% Pluronic F-127 (BASF Corp., Parsippany, NJ) for 1–3 h (11). Fura-2AM and Pluronic F-127 were prepared as stock solutions in DMSO and the total DMSO content that the cells were exposed to was < 0.7%. After fura-2 loading, cells were washed three times with a solution that contained 130 mM NaCl, 3.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1.5 mM KH₂PO₄, 20 mM Hepes, and 10 mM glucose at a pH of 7.40 (solution A). The coverslip was then placed in a plastic cuvette with a coverslip holder designed to hold the coverslip at a 35° angle to the excitation beam (12). The cuvette was placed in a water-jacketed cuvette holder maintained at 37°C. A Perkin-Elmer 650-S10 fluorescent spectrophotometer was used (Perkin-Elmer Corp., Norwalk, CT). Coverslips were superfused at 3 cm³/min with solution A using a peristaltic pump (Buchler Instruments, Fort Lee, NJ). The volume of solution within the cuvette was maintained at 1 cm³. Except where indicated all solutions were equilibrated with room air.

Excitation wavelengths of 340 and 380 nm with a slit width of 1 nm were used. The emission wavelength was 510 nm with a slit width of 10 nm. The following standard formula was used for calculating Ca_i : $Ca_i = K_d [(R - R_{min}) / (R_{max} - R)] [Sf_2 / Sb_2]$ (13, 14). The K_d was assumed to be 224 nM (13). R is the ratio of 510 nm fluorescence at 340 nm excitation over 380 nm excitation. Autofluorescence at 340 and 380 nm was determined using coverslips with cells not loaded with fura-2. These values were subtracted from measured fluorescence before calculation of R . A cell-free solution containing 1 μM fura-2 penta-sodium salt (Molecular Probes), 115 mM KCl, 20 mM NaCl, 10 mM Hepes, and 1 mM MgCl₂ at pH 7.05 and 37°C was used for determination of R_{max} (with 1 mM CaCl₂) and for R_{min} (with 2 mM EGTA). Sf_2 is the fluorescence at 380 nm of the solution used for R_{min} (unbound fura-2). Sb_2 is the fluorescence of the solution used for R_{max} (calcium-bound fura-2). The following values, which were used for the calculation of Ca_i , are the means of 10 determinations: R_{max} , 25.9; R_{min} , 0.93; Sf_2 , 34.3; Sb_2 , 3.4.

Study I

ATP depletion and Ca_i . Fluorescence ratios were recorded every 5 min throughout each experiment. After a 10–20-min equilibration period all experiments followed a similar protocol. There was a 10-min control period followed by a 15-min experimental period during which experimental manipulations were made. Finally, there was a 10-min recovery period when the superfusate was changed back to the control buffer. To facilitate the rapid addition and removal of inhibitors, the extracellular fluid was completely removed and rapidly replaced with the next solution.

To determine whether ATP depletion resulted in a rise in Ca_i , cells were superfused with solution A containing 5 mM sodium cyanide and 5 mM 2-deoxy-D-glucose. Additional experiments used a superfusate of solution A without CaCl₂ and with 2 mM EGTA (solution B). This solution was used to study the effect of Ca_0 removal on Ca_i . solution B with cyanide plus 2-deoxy-D-glucose was used to examine the effect of ATP depletion in the absence of Ca_0 on Ca_i .

To examine whether cyanide and 2-deoxy-D-glucose had effects independent of ATP depletion, experiments were carried out with inhibitors in the presence of glucose. Under these conditions 10 mM glucose allows the production of ATP via glycolysis in spite of 2-deoxy-D-glucose's presence. 1 mM iodoacetate and 10 μM antimycin A were used as an alternative means of inhibiting ATP production. Finally, an oxygen-free environment (100% nitrogen), in the presence of 2-deoxy-D-glucose, was tested to further exclude nonspecific effects of cyanide. This was accomplished by bubbling superfusate with 100%

nitrogen (Medical-Technical Gases Inc., Medford, MA) for 30 min before and throughout the experimental period. During the experimental period air in the cuvette was replaced by a continuous flow of nitrogen.

Study II

Effect of calcium antagonists. Experiments followed a protocol similar to that in study I except that 50 μM 3,4,5-trimethoxybenzoate 8-(*N,N*-diethylamino)octyl ester (TMB-8) and/or 1 μM verapamil were added during control and experimental periods. To further test the role of calcium channels, 100 μM verapamil and 10 μM nifedipine were used. A higher dose of nifedipine could not be used because of its interference with fura-2 fluorescence.

Cell ATP measurements

Cells grown in tissue culture wells were treated with solution A containing metabolic inhibitors using the protocol followed for Ca_i measurement. Experiments with oxygen deprivation were performed by bubbling the solution with 100% nitrogen for 30 min and covering it, while in the tissue culture well, with 1 ml of mineral oil bubbled with 100% nitrogen. Samples were snap frozen with a slurry of isopentane and dry ice. They were then extracted with 4% perchloric acid. ATP content was measured using a modified luciferase assay with purified luciferin/luciferase (Analytical Luminescence Laboratory, San Diego, CA) (15). Protein was assayed by the Coomassie dye method (Bio-Rad Protein Assay; Bio-Rad Laboratories, Richmond, CA).

Statistics

Data are presented as means ± SEM. Statistical significance of results was tested by Student's *t* test for paired and unpaired results. Bonferroni's correction was used for multiple comparisons (16). Significance was defined as a $P < 0.05$.

Results

Baseline Ca_i . Fig. 1 depicts a representative control experiment. There was a constant leak of cellular fura-2 that caused a gradual reduction in total fluorescence. However, because of the continuous exchange of extracellular fluid, the fluorescence ratio, and consequently the measured Ca_i , remained

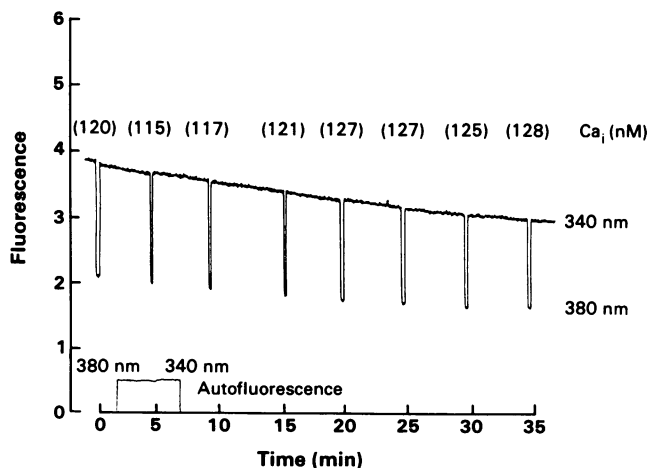


Figure 1. Representative time control experiment. Fluorescence is measured at 510 nm and is presented in arbitrary units. The continuous line is the fluorescence at 340-nm excitation. The lower points are fluorescence measurements at 380-nm excitation taken at 5-min intervals. Actual autofluorescence at 340 and 380 nm is in the lower left corner. Calculated Ca_i values are in parentheses. Ca_i remains unchanged throughout the duration of the experiment.

stable. In 18 control experiments (10 of these are shown in Fig. 6) Ca_i remained stable for the duration of the study.

The Ca_i for all 135 cell monolayers studied was 121 ± 3 nM. The contribution of autofluorescence in 60 monolayers was $9.2 \pm 0.3\%$ for the 340 nm wavelength and $15.1 \pm 0.6\%$ for the 380 nm wavelength. With a superfusion rate of $3 \text{ cm}^3/\text{min}$ the contribution of fluorescence from extracellular fluid (fura-2 leaked from cells) was measured at the 340 nm wavelength during the control period and was found to be $2.7 \pm 0.7\%$ ($n = 7$). None of the substances infused contributed significantly to measured fluorescence at the concentrations used.

Effect of cyanide plus 2-deoxy-D-glucose on cell ATP and Ca_i . A representative experiment of the effect of cyanide plus 2-deoxy-D-glucose in the absence of glucose on Ca_i is shown in Fig. 2. Extrapolation of the 340 nm wavelength signal from the control to the recovery period is consistent with a constant fura-2 leak rate during the experimental period. There was a rise in 340-nm fluorescence and a fall in 380-nm fluorescence that resulted in an increased ratio, which is indicative of a rise in Ca_i . With the change of superfusate back to solution A without the inhibitors there is a rapid reduction of the 340-nm fluorescence and rise of the 380-nm fluorescence. Ca_i returns to baseline within 5 min of recovery. This experiment points out the importance of using the ratio method of calculating Ca_i because it corrects for changes in fura-2 concentration. If only the 340-nm wavelength was used, the rise in Ca_i would be underestimated and the calculated recovery of Ca_i would be too low. Fig. 3 depicts the results of 11 experiments. Ca_i rose from 112 ± 11 nM during C-10 to 649 ± 99 nM after 15 min of exposure to cyanide plus 2-deoxy-D-glucose without glucose (E-15), and returned to 101 ± 16 nM after 10 min of recovery (R-10). The Ca_i at E-15 was significantly greater than the values at C-10 and R-10 ($P < 0.0005$ in each case). Three experiments were carried out with the identical protocol except that the cells were not loaded with fura-2. Cyanide and

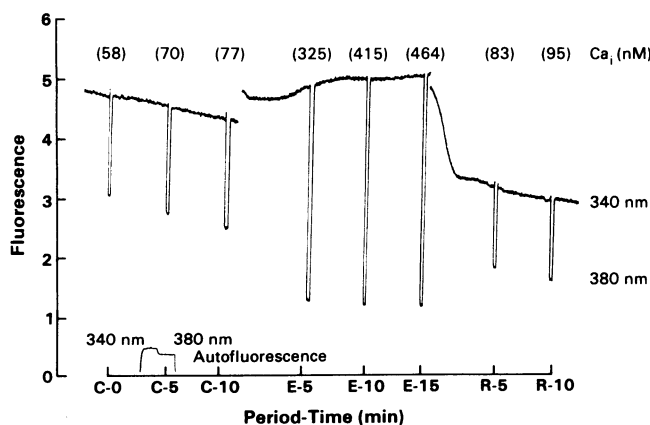


Figure 2. Representative experiment of the effect of cyanide and 2-deoxy-D-glucose on Ca_i . The format is the same as in Fig. 1. On the abscissa C, E, and R represent control, experimental, and recovery periods, respectively. Fluorescent ratios were measured at 5-min intervals. During the 15-min experimental period the superfusate contained cyanide and 2-deoxy-D-glucose without glucose. Immediately after the E-15 measurement, the superfusate is changed to the control solution for the 10-min recovery period. Ca_i rises during the experimental period and recovers rapidly with removal of cyanide and 2-deoxy-D-glucose.

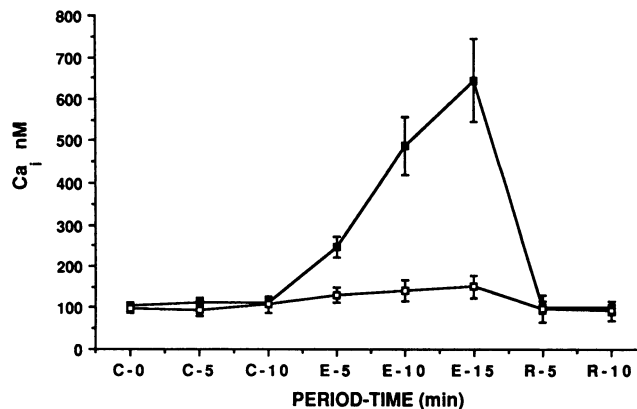


Figure 3. The effect of cyanide and 2-deoxy-D-glucose with and without Ca_0 on Ca_i . With Ca_0 present (closed squares; $n = 11$) Ca_i is significantly greater at E-15 than at C-10 ($P < 0.001$). At period E-15 those cells exposed to inhibitors in the absence of Ca_0 (open squares; $n = 6$) had a significantly lower Ca_i than those exposed to inhibitors in the presence of Ca_0 ($P < 0.005$).

2-deoxy-D-glucose had no effect on the autofluorescence of these cells.

Baseline cell ATP content of MDCK cells grown in monolayers was 23.5 ± 5.0 nmol/mg protein. With exposure to cyanide plus 2-deoxy-D-glucose in the absence of glucose, ATP fell progressively over 15 min. Expressed as percent of control, ATP content, cell ATP was 37 ± 6 , 19 ± 3 , and $11 \pm 2\%$ at 5, 10, and 15 min, respectively (Fig 4). Recovery of ATP levels after the removal of cyanide plus 2-deoxy-D-glucose was rapid but incomplete, reaching 52 ± 8 and $42 \pm 8\%$ of control at 5 and 10 min of the recovery period, respectively. Control, E-15, and R-10 values are significantly different from each other ($P < 0.001$ in each case).

Experiments carried out with cyanide and 2-deoxy-D-glucose in the presence of 10 mM glucose produced no rise in Ca_i (Table I). Under these conditions ATP levels were $61 \pm 5\%$ of control values. Using iodoacetate and antimycin A to inhibit ATP production Ca_i rose from 112 ± 16 to 367 ± 27 nM and

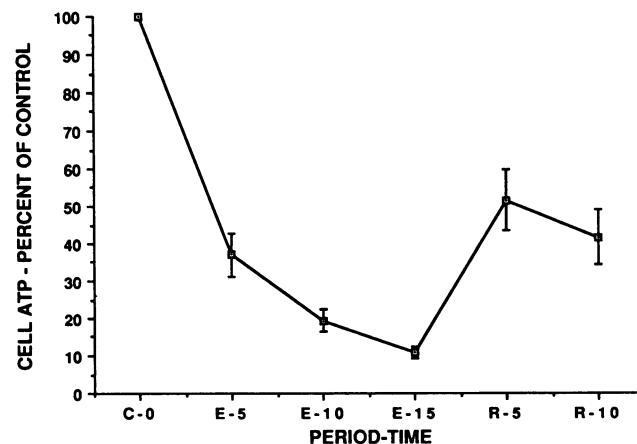


Figure 4. The effect of cyanide and 2-deoxy-D-glucose on cell ATP. ATP is expressed as percent of control (C-0). C-0, E-15, and R-10 are significantly different from each other at $P < 0.001$ for each comparison ($n = 12$).

Table I. The Effects of 15 min of Exposure to Metabolic Inhibitors on Cell ATP and Ca_i

	5 mM cyanide, 5 mM 2-deoxy-D-glucose, and no glucose	5 mM cyanide, 5 mM 2-deoxy-D-glucose, and 10 mM glucose	1 mM iodoacetate and 10 μ M antimycin A	5 mM 2-deoxy-D-glucose, 100% nitrogen, and no glucose	100% nitrogen and no glucose
ATP (% control)	11 \pm 2 (12)	61 \pm 5 (8)	—* (8)	9 \pm 3 (8)	50 \pm 7 (6)
Ca_i (nM)	649 \pm 99 (11)	107 \pm 15 (6)	367 \pm 27 (6)	924 \pm 142 (6)	121 \pm 12 (6)

The number in parentheses denotes the number of experiments in each group. * Undetectable.

continued to rise after the removal of inhibitors to 605 \pm 84 nM (Table I). These inhibitors irreversibly reduced ATP to undetectable levels.

Cells exposed to an oxygen-free (100% nitrogen) and glucose-free solution containing 2-deoxy-D-glucose had a fall in ATP to 9 \pm 3% of baseline and a rise in Ca_i from 165 \pm 25 to 924 \pm 142 nM. Identical experiments carried out in the presence of glucose produced no rise in Ca_i and ATP was 50 \pm 7% of baseline.

Ca_o influx and Ca_i redistribution. To examine the role of calcium influx during ATP depletion, MDCK cells were studied in the absence of Ca_o and glucose with cyanide plus 2-deoxy-D-glucose (solution B with cyanide plus 2-deoxy-D-glucose). Instead of an increase from 112 \pm 11 to 649 \pm 99 nM with Ca_o present (Fig. 3), the rise induced by metabolic inhibitors in the absence of Ca_o went from 108 \pm 21 to 151 \pm 28 nM (Figs. 3 and 5). Thus, the removal of Ca_o significantly blunted the rise in Ca_i ($P < 0.005$), which demonstrates the important role of calcium influx during ATP depletion.

However, even in the absence of Ca_o , Ca_i rose significantly ($P < 0.01$), which suggests that Ca_i redistribution contributes to the rise in Ca_i induced by cyanide plus 2-deoxy-D-glucose. This result is emphasized by the finding that removal of Ca_o alone (Fig. 5) causes a reduction in Ca_i from 127 \pm 7 to 38 \pm 6 nM ($P < 0.005$). This confirms that redistribution of Ca_i to the cytosolic compartment contributes to the rise in Ca_i with ATP depletion.

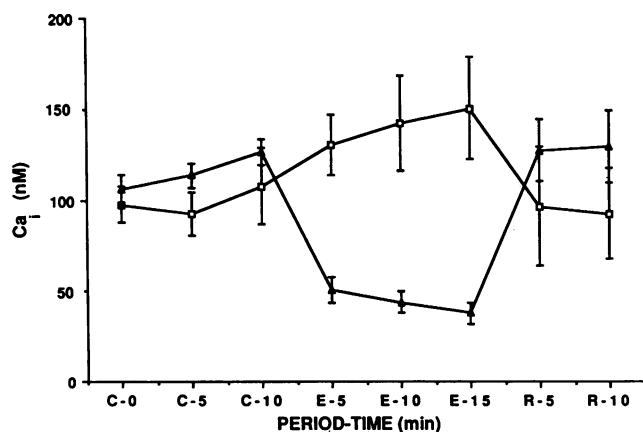


Figure 5. The effect of Ca_o removal with and without cyanide and 2-deoxy-D-glucose on Ca_i . With Ca_o removal and exposure to inhibitors (open squares, same data as Fig. 3, $n = 6$) Ca_i rises (C-10 vs. E-15) ($P < 0.01$). Removal of Ca_o (closed triangles) results in a reduction of Ca_i (C-10 vs. E-15) ($P < 0.005$). At E-15 the Ca_i is significantly different between the two manipulations ($P < 0.005$). Note that the scale is different from that in Fig. 3 and 6.

Effect of TMB-8 and/or calcium channel blockers on the cyanide plus 2-deoxy-D-glucose-induced rise in Ca_i . Neither TMB-8 nor verapamil had a consistent effect on baseline Ca_i . After pretreatment with 1 μ M verapamil, exposure to cyanide plus 2-deoxy-D-glucose resulted in a rise in Ca_i from 103 \pm 10 to 605 \pm 103 nM ($P < 0.0005$), which returned to 72 \pm 4 nM during the recovery period (Fig. 6 A). This rise was not signifi-

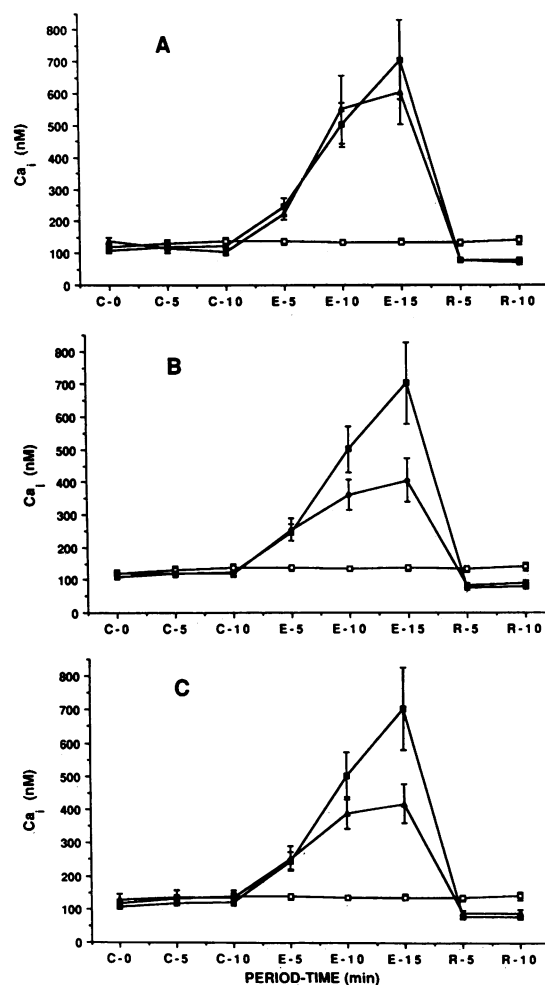


Figure 6. The effect of TMB-8 and verapamil on the rise of Ca_i produced by cyanide and 2-deoxy-D-glucose. Time control (open squares; $n = 10$) and cyanide and 2-deoxy-D-glucose (closed squares; $n = 20$) experiments are the same for all three panels. (A) Verapamil (open triangles; $n = 10$) did not effect the increase in Ca_i . (B) TMB-8 (open diamonds; $n = 10$) (C) TMB-8 with verapamil (closed triangles; $n = 10$) did blunt the rise in Ca_i induced by cyanide and 2-deoxy-D-glucose. TMB-8 significantly blunted the rise in Ca_i ($P < 0.01$).

cantly different from that produced by the inhibitors alone (122 ± 9 to 705 ± 125 nM). Expressed as the percent of baseline values, the increase in Ca_i with cyanide plus 2-deoxy-D-glucose was 494%, and with 1 μ M verapamil and cyanide plus 2-deoxy-D-glucose it was 501%. At higher concentrations of verapamil (100 μ M), Ca_i rose from 155 ± 23 to 943 ± 165 nM, with 10 μ M nifedipine the rise was from 181 ± 28 to 924 ± 213 nM, and in concurrent experiments without calcium antagonists Ca_i rose from 130 ± 20 to 887 ± 360 nM ($n = 6$ for each group).

In the presence of TMB-8, cyanide plus 2-deoxy-D-glucose without glucose caused a rise in Ca_i from 121 ± 11 to 406 ± 67 nM (Fig. 6 B). With TMB-8 and 1 μ M verapamil present, exposure to cyanide plus 2-deoxy-D-glucose resulted again in a significant rise in Ca_i that went from 133 ± 24 to 419 ± 59 nM ($P < 0.005$; Fig. 6 C). The peak values of Ca_i produced by metabolic inhibitors in the presence of TMB-8 or TMB-8 plus verapamil (406 ± 67 and 419 ± 59 nM, respectively) were not different. Verapamil had no effect on the rise of Ca_i produced during ATP depletion. Therefore, for statistical purposes we combined all 20 experiments in which TMB-8 was used and found that the rise in Ca_i induced by cyanide plus 2-deoxy-D-glucose was significantly blunted by TMB-8 ($P < 0.01$).

Discussion

Our data demonstrate that depleting cultured renal epithelial cells of ATP results in nearly a 500% rise in Ca_i . In the absence of glucose, 5 mM 2-deoxy-D-glucose and 5 mM sodium cyanide produced a rapid reduction in cell ATP that was quantitatively similar to that measured in other models of renal ischemia (17, 18). These inhibitors did not produce a rise in Ca_i in the presence of glucose. Furthermore, iodoacetate and antimycin A, as well as 2-deoxy-D-glucose in the absence of oxygen and glucose, can produce an ATP depletion large enough to cause a rise in Ca_i . Therefore, the rise in Ca_i was indeed due to ATP depletion and not to other effects of cyanide or 2-deoxy-D-glucose.

The experimental techniques used are the pharmacological equivalents of substrate removal and hypoxia. In the absence of glucose, 2-deoxy-D-glucose inhibits glycolysis and sodium cyanide inhibits mitochondrial ATP production. In this model not all of the aspects of *in vivo* renal ischemia are reproduced (e.g., extracellular acidosis does not develop and accumulation of metabolic by products does not occur). These other events are not necessary for a rise in Ca_i to take place.

The rise in Ca_i that occurred with exposure to cyanide plus 2-deoxy-D-glucose in the absence of glucose had a similar time course as the reduction in ATP. There was a progressive increase in Ca_i over the 15-min experimental period. Not only is the maintenance of basal Ca_i dependent on ATP, but it appears that a threshold level of ATP content may exist since Ca_i was unchanged when ATP was reduced to 50% of baseline. It is interesting to note that in this cell line, ATP levels remain adequate to maintain basal Ca_i concentrations for 15 min in the absence of glucose and oxygen. The presence of the glycolytic inhibitor 2-deoxy-D-glucose is required to further reduce ATP levels until they fall below the threshold necessary for the maintenance of basal Ca_i . This suggests that endogenous substrates exist that can be used to produce ATP via glycolysis.

These results suggest that ATP depletion disrupts normal cell calcium homeostasis. ATP is needed to provide energy for calcium extrusion from the cell whether it occurs directly by

the plasma membrane calcium ATPase or indirectly by the Na^+/Ca^{++} exchanger (3, 4). Uptake of calcium into endoplasmic reticulum is also energy dependent and prevented by ATP depletion. Mitochondrial function, and consequently calcium uptake, would be impaired also in this model as it would be during ischemia. Additionally, calcium leak into cells may be accelerated during ischemia because of an impaired maintenance of plasma membrane integrity. Accumulation of abnormal lipids in the plasma membrane has been described previously in renal ischemia and these may act as calcium ionophores (19). Metabolism of these lipids is likely to be an energy-dependent process that does not occur during ATP depletion. It does not appear that oxygen-free radicals mediate the rise in Ca_i in this system since it occurs in experiments where oxygen is absent (100% nitrogen).

A rise in Ca_i of the magnitude we found has not been reported previously to occur during cellular ATP depletion or ischemia. Snowdowne and co-workers have examined the effect of hypoxia and substrate depletion on Ca_i in cultured renal cells as well as in cardiac myocytes (20, 21). In their studies substrate depletion and hypoxia produced a rise in Ca_i of 100–200%. They used aequorin to measure Ca_i , which requires exposure of the cells to hypotonic solutions so that the calcium-sensitive probe may gain entry into the cell. Whether this loading technique affected their results is unknown. Others have not measured a rise in Ca_i in models that simulate cell ischemia in erythrocytes or in cardiac myocytes (22, 23). Lemasters et al., using cultured hepatocytes, did not demonstrate any rise in Ca_i as measured with fura-2 upon exposure to 5 mM cyanide and 10 mM iodoacetate (24). Though ATP content was not measured, this combination should adequately inhibit ATP production. There was a loss of mitochondrial membrane potential difference and cell blebbing during the initial 30 min, and after 60 min there was cell death. It is difficult to reconcile the lack of a rise in Ca_i with the morphological changes seen in the plasma membrane. These changes should result in increased permeability and influx of calcium into the cell. Indeed, during the terminal phases of the study, Ca_i is reported as unchanged despite fura-2 leakage from cells and leakage of the cationic dye, propidium, into the cells.

Most of the evidence suggesting that a rise in Ca_i occurs during ischemia has been indirect. Total cell calcium content as well as mitochondrial calcium content have been shown to rise during ischemia, suggesting an influx of Ca_0 (1, 2, 7–9). Calcium channel blockers have been inconsistent in protecting against ischemia in the isolated perfused kidney preparation (25, 26). In cultured renal epithelial cells subjected to substrate depletion and hypoxia, verapamil and nifedipine improved cell viability (27). However, these drugs did not protect isolated cardiac myocytes from hypoxia and substrate depletion (28). This indirect evidence has been inconclusive regarding a rise in Ca_i during ischemia or ATP depletion.

Differences in cell type and experimental methodologies might explain the varied results cited above. However, the mechanisms by which a cell defends itself against a rise in Ca_i are considered to be similar in all cells (1, 3). Regardless of variability among cell types, the maintenance of a 10,000-fold difference between Ca_0 and Ca_i requires energy in the form of ATP. Depletion of ATP should result in a dissipation of this gradient and our data clearly demonstrate this to be true.

It appears that an influx of Ca_0 contributes to this increment. In the absence of Ca_0 , Ca_i rises only a small amount

with exposure to cyanide plus 2-deoxy-D-glucose, in contrast to the nearly fivefold rise seen in the presence of Ca_0 . Exposure of cells to a calcium-free environment may deplete intracellular stores of calcium. However, cells were not exposed to calcium-free perfusate until metabolic inhibitors were added, which thus minimized calcium depletion. That calcium from intracellular stores also contributes to this rise is shown by the different response of Ca_i to the removal of Ca_0 with and without cyanide plus 2-deoxy-D-glucose. Removal of Ca_0 results in a 70% reduction in Ca_i . In the presence of cyanide plus 2-deoxy-D-glucose, but without Ca_0 , the Ca_i rises 46%. The contribution of intracellular stores is further supported by the ability of TMB-8 to blunt the rise in Ca_i induced by the inhibitors. While the primary action of TMB-8 is to inhibit the release of calcium from intracellular stores in some cell types, it may also block calcium influx (30).

To examine the role of voltage-dependent calcium channels, the effect of verapamil was studied. During cell ATP depletion, the Na^+/K^+ ATPase loses its energy source. Consequently, the negative cell potential is not maintained and the cell depolarizes. This could activate calcium channels if they are present. Treatment with 1 or 100 μM verapamil, as well as 10 μM nifedipine, did not affect the rise in Ca_i induced by ATP depletion. This was true for 1 μM verapamil even when TMB-8 was present to prevent Ca_i redistribution, and thus to allow any small contribution through voltage-dependent channels to be measured. These results suggest that voltage-dependent channels are not responsible for the calcium influx during ATP depletion in MDCK cells. The in vivo efficacy of calcium channel blockers may not be due to effects on renal epithelial cells but on vascular smooth muscle cells or on calcium accumulation during the reflow phase after ischemia.

The study of cells in confluent monolayers offers some advantages over studying cells in suspension. Epithelial cells develop polar transport characteristics when grown in monolayers that are lost when cells are in suspension. This is not likely to be important in our experiments but may affect the results of experiments involving cell transport. To place cells in suspension they must be treated with trypsin in a magnesium- and calcium-free solution and/or scraped from the substratum upon which they grow. Cell permeability to fura-2 and calcium may be affected by these maneuvers. Most importantly, having the cells adhere to a coverslip within the cuvette allows for a continuous exchange of extracellular fluid. Fura-2 leaked from cells can be continuously removed, so that it would result in a negligible contribution to the total fluorescence from extracellular dye. The addition of EGTA or manganese is not necessary to quench extracellular fluorescence. Continuous superfusion of the extracellular fluid permits multiple fluid exchanges without the manipulation of the cells on the coverslip.

One advantage of fura-2 over its predecessor, quin 2, is the use of a fluorescence excitation ratio to calculate Ca_i . This theoretically improves sensitivity since both wavelengths (340 and 380 nm) are responsive to changes in Ca_i . The ratio method of calculating Ca_i is particularly important because over the duration of these experiments there is a significant leak of fura-2 (see Figs. 1 and 3). If only the 340-nm wavelength were used a reduction in fluorescence would be interpreted as a reduction in Ca_i and not as a leak of indicator.

These experiments demonstrate, for the first time, that inhibition of cellular ATP production results in a nearly 500%

rise in Ca_i . Influx of Ca_0 as well as the release of calcium from intracellular sites appear to contribute to this rise. Verapamil and nifedipine have no effect on the rise in cytosolic calcium produced by metabolic inhibitors in this cell line. TMB-8, which inhibits the release of calcium from intracellular stores, reduced the increment in Ca_i that resulted from ATP depletion. A rise in Ca_i , as seen in this model, may also occur in ischemic acute renal failure as a consequence of ATP depletion. Because there are multiple sources of this calcium, it is unlikely that a calcium antagonist with a single mechanism of action could totally ameliorate the increase in Ca_i .

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