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Research Article

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Cytosolic-free Calcium Increases to Greater Than 100 Micromolar in ATP-depleted Proximal Tubules

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Abstract

Previous studies have shown that cytosolic-free Ca^{2+} (Ca_f) increases to at least low micromolar concentrations during ATP depletion of isolated kidney proximal tubules. However, peak levels could not be determined precisely with the Ca^{2+}-sensitive fluorophore, fura-2, because of its high affinity for Ca^{2+}. Now, we have used two low affinity Ca^{2+} fluorophores, mag-fura-2 (furaptra) and fura-2FF, to quantitate the full magnitude of Ca_f increase. Between 30 and 60 min after treatment with antimycin to deplete ATP in the presence of glycine to prevent lytic plasma membrane damage, Ca_f measured with mag-fura-2 exceeded 10 μM in 91% of tubules studied and 68% had increases to greater than 100 μM. Ca_f increases of similar magnitude that were dependent on influx of medium Ca^{2+} were also seen using the new low Ca^{2+} affinity, Mg^{2+}-insensitive, fluorophore fura-2FF in tubules depleted of ATP by hypoxia, and these increases were reversed by reoxygenation. Total cell Ca^{2+} levels in antimycin-treated or hypoxic tubules did not change, suggesting that mitochondria were not buffering the increased Ca_f during ATP depletion. Considered in the context of the high degree of structural preservation of glycine-treated tubule cells during ATP depletion and the commonly assumed Ca^{2+} requirements for phospholipid hydrolysis, actin disassembly, and Ca^{2+}-mediated structural damage, the remarkable elevations of Ca_f demonstrated here suggest an unexpected resistance to the deleterious effects of increased Ca_f during energy deprivation in the presence of glycine. (*J. Clin. Invest.* 1997. 100:713–722.) Key words: hypoxia • glycine • mag-fura-2 • fura-2FF • kidney

Introduction

The mechanisms responsible for damage and disintegration of energy deprived cells have remained elusive despite much study. Deregulation of cellular Ca^{2+} homeostasis is among the processes potentially of importance. Multiple factors favor the increase of cytosolic-free Ca^{2+} (Ca_f)[1] during injury, and increased Ca_f is known to catalyze the breakdown of membranes and cytoskeletal structures by hydrolysis and disassembly (1–4). An understanding of these processes requires that abnormalities of Ca^{2+} homeostasis during cell injury be accurately characterized. The difficulties inherent in measuring Ca_f are compounded by injury states and considerable controversy remains about the pathogenetic role of Ca_f alterations (1–8).

Because of its structural complexity, fragility, and highly active transport systems, the proximal tubule has posed special challenges to assessment of Ca_f and its role during injury. Damage to this nephron segment is of particular significance to kidney function during acute renal failure and the ability to readily isolate it provides opportunities to study injury processes of general interest in a well-preserved, highly differentiated epithelial cell in vitro. Recent observations have led to the refinement of methodology and an improved conceptual framework to address questions of mechanistic significance relevant to the role played by Ca_f in proximal tubule damage. The recognition that simple restoration of glycine that is lost from cells during ATP depletion confers a high degree of resistance to lytic membrane damage has been particularly important in this regard and allows study of the full progression of prelethal injury and the biochemical events responsible for it in a fashion uncomplicated by development of nonspecific changes due to generalized cellular disruption (9–17). The 2–5 mM concentrations of glycine that confer protection when added to the medium of ATP-depleted, isolated tubules increase intracellular glycine to the same levels, which are at the low end of the range of renal cortex glycine concentrations measured in vivo in various species (11).

Glycine-replete tubules have been shown to be resistant to lytic membrane damage produced by extreme perturbations of Ca_f induced by treatment with the ionophore, ionomycin, in combination with either the metabolic transport inhibitor, antimycin (13–16), or various uncouplers (12, 17, 18). However, such tubules develop severe structural alterations associated with Ca^{2+}-dependent phospholipid hydrolysis (13, 15, 19) and actin disassembly (16, 20). The use of glycine has also led to the recognition that sustained increases of Ca_f to at least low μM concentrations as measured by fura-2 develop during ATP depletion produced by hypoxia (21–24), antimycin (13, 19), or uncouplers (12, 17) without ionophore. However, under these conditions, structural damage in glycine-protected cells is less (10, 13, 16, 25) and phospholipid hydrolysis (15) and actin cy-

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1. *Abbreviations used in this paper:* Ca_f, cytosolic-free Ca^{2+}; EDTA, ethylenediaminetetraacetate; EGTA, ethyl glycol-bis (β-amino-ethyl-ether); FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; LDH, lactate dehydrogenase; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one[bis(hexafluoroacetonyl)acetone; Rmax, ratio measured during perfusion with ionomycin in 3.5 mM Ca^{2+} solution; Rmin, ratio measured during perfusion with EGTA and ionomycin in Ca^{2+}-free solution.

toskeletal alterations (16) are largely Ca^{2+}-independent, thus bringing into question the true magnitude and significance of the Ca_f increases measured with the fluorophore.

To the extent that they have been defined thus far, critical Ca^{2+} mediated events in ionophore-treated, ATP-depleted tubules have required Ca^{2+} concentrations of ≥ 10 μM (15, 20). Because of its high affinity for Ca^{2+}, fura-2 does not accurately estimate Ca_f at concentrations exceeding 1 μM (26). Mag-fura-2 (furaptra) was introduced as a fluorophore suitable for assessing intracellular free Mg^{2+}, although it was recognized from the outset that the dye is also highly Ca^{2+} responsive (27). The affinity of mag-fura-2 for Ca^{2+} is substantially greater than its affinity for Mg^{2+}, but much less than the affinity of fura-2 for Ca^{2+} (27–29). This property has allowed use of mag-fura-2 in a number of systems to quantify Ca_f increases well into the μM range (28, 30–33). Mag-fura-2 has also been used under conditions of ATP depletion to assess release of Mg^{2+} associated with hydrolysis of MgATP (33–36). During studies originally designed to assess Mg^{2+}, we noted that mag-fura-2 fluorescence changes in ATP-depleted, isolated proximal tubules reflected increases of Ca_f to the μM range with little or no interference from concomitant alterations of Mg^{2+}. In this report we have corroborated this observation and used the method to assess the full extent of Ca_f increase in ATP-depleted proximal tubules. The results indicate that sustained increases of Ca_f to values that exceed 100 μM develop in the majority of tubules subjected to $>$ 30 min antimycin-induced ATP depletion in the presence of glycine without evidence for lytic plasma membrane damage as indicated by accelerated fluorophore leakage or failure of vital dye exclusion. Moreover, we demonstrate similar increases of Ca_f in glycine-protected hypoxic tubules that reverse with reoxygenation using a newly available, alternative, low affinity Ca^{2+} fluorophore, fura-2FF. Prelethal increases of Ca_f of this magnitude have never been recognized previously in any cell type. Considered in light of the information on Ca^{2+} dependence of phospholipid and cytoskeletal alterations (13, 15, 16, 20) and the remarkable retention of structural integrity of glycine-protected cells during ATP depletion (10, 13, 20), these data suggest exclusion of some cellular compartments from the increases of Ca_f and/or an unexpectedly high resistance to the deleterious effects of Ca^{2+} in glycine-protected, ATP-depleted cells.

Methods

Isolation of tubules. Proximal tubules were prepared from kidney cortex of female New Zealand white rabbits (1.5–2.0 kg; Oakwood Farms, Oakwood, MI) by collagenase digestion and centrifugation on self-forming Percoll gradients (9, 11, 12). Final tubule pellets were resuspended at a concentration of 4–5 mg tubule protein per milliliter in ice-cold medium gassed with 95% O_2/5% CO_2. The medium contained (in mM) 105 NaCl, 2.6 KCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $MgSO_4$, and, for most studies, 2.5 probenecid to promote intracellular retention of the fluorophores (12, 14). This medium also included (in mM) 5 glucose, 4.5 sodium lactate, 1 alanine, and 10 sodium butyrate (Solution A).

Loading of tubules with mag-fura-2/acetoxymethylester (AM). To load cells with mag-fura-2 (Molecular Probes, Inc., Eugene, OR) or fura-2FF (TeFlabs, Austin, Texas), 25 μl of 1.0 mM stock solutions of the acetoxymethylester forms in dimethyl sulfoxide were mixed vigorously into 500 μl of medium without tubules and the mixture was then added to 4.5 ml of the tubule suspension to produce a 5 μM final concentration. The siliconized, 25 ml Erlenmeyer flask containing these tubules was gassed with 95% O_2/5% CO_2, sealed, and shaken gently in a water bath at 25°C for 60 min. The tubules were then pelleted by centrifugation at 30 g, washed twice in ice-cold medium containing (in mM) 110 NaCl, 3.5 KCl, 1.0 KH_2PO_4, 1.25 $CaCl_2$, 1.0 $MgCl_2$, and 25 Na-Hepes, pH 7.2, and then resuspended in this medium (Solution B). 500-μl aliquots of this suspension were applied to 25 mm diameter #1 1/2 glass coverslips (Nicholson Precision Instruments, Gaithersburg, MD) that had been precoated with poly-D-lysine. The coverslips were kept in covered 35-mm culture dishes at 4°C for a minimum of 20 min to allow maximal settling and adherence of tubules before study.

Fluorescence measurements in superfused tubules. A coverslip with adherent tubules was mounted in a Dvorak-Stotler culture chamber (Nicholson Precision Instruments) and perfusion at 0.82 ml/min from a 50-ml syringe, using a Harvard Apparatus, Inc. (South Natick, MA) Model 975 pump, was started immediately with a solution similar to Solution A except that the alanine concentration was reduced to 0.3 mM, the sodium butyrate concentration was reduced to 5 mM, 2 or 5 mM glycine was present, and the Ca^{2+} and Mg^{2+} concentrations were adjusted as indicated for specific experiments (Solution C). The microfluorometric measurements were made using a Nikon Diaphot inverted microscope and a SPEX Industries (Edison, New Jersey) CM-2 spectrofluorometer system with the DM 3000 computer and software. The chamber and microscope stage were kept at 37°C using an Air Stream Incubator (Nicholson Precision Instruments). The tubule was viewed using a CF FLUOR 100× Oil/Iris lens (Nikon, Garden City, NY) and was illuminated alternately with 340- and 380-nm excitation signals for 0.33 s through 0.4 mm slits ahead of the chopper, an ND0.3 neutral density filter (Omega Optical, Brattleboro, VT), and a DM 400 dichroic mirror (Nikon). Emitted light was collected through the dichroic mirror and then transmitted via a 420BA filter in the microscope and a 500FS40 filter (SPEX Industries) in the photomultiplier assembly to the fluorometer photomultiplier.

Mounting the chamber on the microscope stage, locating and positioning a tubule using visible light from the microscope, and making final adjustments to perfusion syringes and experimental parameters on the computer generally required 5–7 min during which the coverslip was perfused with Solution C at 37°C. An additional period of 10–15 min perfusion with Solution C was then recorded before experimental maneuvers were started.

Experimental agents were introduced by replacing the perfusion syringe with one containing a solution of the desired composition, i.e., 95% O_2/5% CO_2–gassed Solution C with ATP-depleting metabolic inhibitor and various Ca^{2+} concentrations or, for hypoxia studies, 95% N_2/5% CO_2–gassed Solution C containing 1800 mU/ml of Oxyrase (Oxyrase Inc., Mansfield, Ohio) to maximize the degree of hypoxia (37). Reoxygenation after hypoxia was done with 95% O_2/5% CO_2–gassed Solution C that was further supplemented with 4 mM each of α-ketoglutarate and aspartate (38) and 250 μM MgATP (39) to promote rapid recovery of cell ATP. In some experiments, after ATP depletion or reoxygenation, tubules were superfused with 1 μM antimycin plus 15 μM ionomycin in 10, 50, or 100 μM Ca^{2+} medium (prepared using EGTA/Ca^{2+}–buffered solutions as in reference 20) to provide an additional internal calibration point for the response of each tubule at these Ca^{2+} levels spanning the response range of greatest interest. After the last desired experimental maneuver, tubules were subjected to a terminal calibration procedure consisting of perfusion with a nominally Ca^{2+} and/or Mg^{2+}-free form of Solution C that also contained 2.5 mM ethylene glycol-bis(β-amino-ethyl-ether) (EGTA) or 2.5 mM ethylenediaminetetraacetate (EDTA) and 15 μM ionomycin or 15 μM bromo-A23187 (Calbiochem Corp., La Jolla, CA), followed by Solution C supplemented with Ca^{2+} to 3.5 mM or Mg^{2+} to 20 mM and 15 μM ionomycin or 15 μM 4-bromo-A23187, then by Solution C containing 200 μM $MnCl_2$. Each of the calibration solutions was continued until the response was stable. Glycine at 2 or 5 mM concentrations was present in all experimental and calibration solutions. This completely prevents lytic membrane damage and the associated accelerated dye leakage under these ex-

perimental conditions (12, 13, 15). As we have observed previously for fura-2 (12), superfusion with 20 μM digitonin rapidly decreased mag-fura-2 and fura-2FF fluorescence by > 90% indicating predominant cytosolic localization of both fluorophores.

For quantitation of Ca_f, the values of the 340 and 380 nm signals at the end of the $MnCl_2$ perfusion were subtracted to correct for autofluorescence and other Ca^{2+}-independent fluorescence in the system (12). Ca_f was calculated from the background-corrected 340/380 ratios using the equation:

$$[Ca^{2+}]_f = (K_d \cdot S_{f2}/S_{b2}) \cdot (R - Rmin) / (Rmax - R)$$

where *R* is the ratio at any point; *Rmin* is the ratio measured during perfusion with EGTA and ionomycin in Ca^{2+}-free solution C; *Rmax* is the ratio measured during perfusion with ionomycin in 3.5 mM Ca^{2+} solution C; Sf_2 is the absolute value of the corrected 380 signal at Rmin; Sb_2 is the absolute value of the corrected 380 signal at Rmax. A K_d of 33 μM for Ca^{2+} was measured as described in Results for intracellular mag-fura-2 and the K_d of fura-2FF was similarly measured to be 13 μM. These values were used in all calculations of Ca_f. No attempt was made to mathematically correct for fluorescence secondary to concomitant changes of $[Mg^{2+}]$ in the mag-fura-2 studies because such changes were either absent or small under the experimental conditions (see Results).

Measurement of total cell Ca^{2+} and lytic damage in suspended tubules. Flasks containing tubules in Solution A without probenecid were incubated at 37°C for 15 min in the metabolic shaker. Then, experimental additions were made, flasks were regassed with 95% O_2/5% CO_2 or, for hypoxia studies, 95% N_2/5% CO_2, and were incubated for 15–60 min before sampling. Total cell Ca^{2+} was measured by atomic absorption spectroscopy in tubules separated from their medium by centrifugation through bromododecane (Aldrich Chemical Co., Milwaukee, WI) and was factored by cell protein measured by the Lowry method as described previously (9). Lactate dehydrogenase (LDH) was measured in the medium before and after the addition of 0.1% Triton X-100 (40).

Reagents. Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis(trifluoromethyl)heptan-4-one[bis(hexafluoroacetonyl)acetone(1799), a nonfluorescent uncoupler of mitochondrial oxidative phosphorylation (41), was provided by Dr. Peter Heytler (DuPont, Wilmington, DE). 1799 or an alternative low fluorescence uncoupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), antimycin, and ionomycin were delivered from 1,000× absolute ethanol stocks. Free Ca^{2+} levels in reduced Ca^{2+} experimental solutions were verified with the pentapotassium salts of either fura-2 (solutions with < 1 μM free Ca^{2+}) or calcium orange-5N (solutions with > 3–5 μM Ca^{2+}) as most appropriate (20).

Statistics. Data from multigroup experiments were analyzed statistically by analysis of variance for repeated measure designs. Individual group comparisons were then made using the Neuman-Keuls test for multiple comparisons (SigmaStat; Jandel Scientific, San Rafael, CA). Two group studies were assessed using paired or unpaired *t* tests as appropriate. $P < 0.05$ was considered to be statistically significant.

Results

Fig. 1 illustrates representative tracings from the initial experiments assessing fluorescence of mag-fura-2-loaded tubules subjected to ATP depletion induced by either the electron transport inhibitor, antimycin, or the nonfluorescent uncoupler, 1799, in the presence of glycine. Some tubules such as those shown in *A* and *B* developed small increases of the 340/380 ratio that were then returned to below the baseline by treatment with A23187 and EDTA in Ca, Mg-free medium. Further superfusion with 20 mM Mg^{2+} in Ca-free medium in

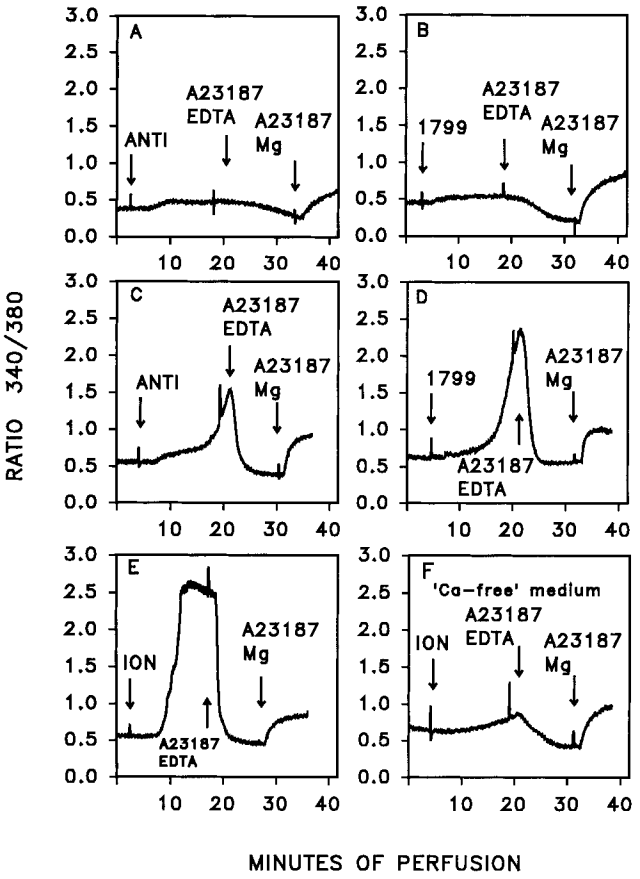

Figure 1. Initial studies of fluorescence changes in mag-fura–loaded tubules during ATP depletion conditions. Tubules were superfused for a baseline period in Solution C containing 1.25 mM Ca^{2+} and 2.4 mM Mg^{2+} with 2 mM glycine followed by introduction in the same medium of either 1 μM antimycin (*ANTI*) (*A* and *C*), 20 μM 1799 (*B* and *D), 15* μM ionomycin (*ION*) (*E*) or 15 μM ionomycin in Ca-free medium (*F*). After 15 min with the experimental agent, perfusion was switched to Ca,Mg-free medium containing 2.5 mM EDTA and 15 μM 4-bromo-A23187, which was followed by Ca-free medium supplemented with 20 mM Mg^{2+} and 15 μM 4-bromo-A23187. A final perfusion with Ca,Mg-free medium containing 200 μM $MnCl_2$ to quench mag-fura fluorescence for determination of background autofluorescence is not shown on the tracings. Values given are the ratios of the signals measured during alternate excitation at 340 and 380 nm.

the continued presence of A23187 then produced a moderate increase of the fluorescence ratio. Other tubules, however, such as those shown in *C* and *D*, developed large increases of the fluorescence ratio toward the end of the predefined period of treatment with the ATP-depleting agent. The increases substantially exceeded the values measured in the presence of 20 mM Mg^{2+} and A23187. Because the timing of these changes in fluorescence was similar to those seen with the Ca^{2+} indicator, fura-2 (12), and, in view of the reported sensitivity of mag-fura-2 to Ca^{2+} (27, 28, 30–33), we then tested the effect of introducing ionomycin during the initial period of superfusion with regular medium containing Ca^{2+} and Mg^{2+}. A large increase of fluorescence was seen (*E*) and this increase was blocked if Ca^{2+} was removed from the medium despite continued superfusion with Mg^{2+}. Consistent with the presence of

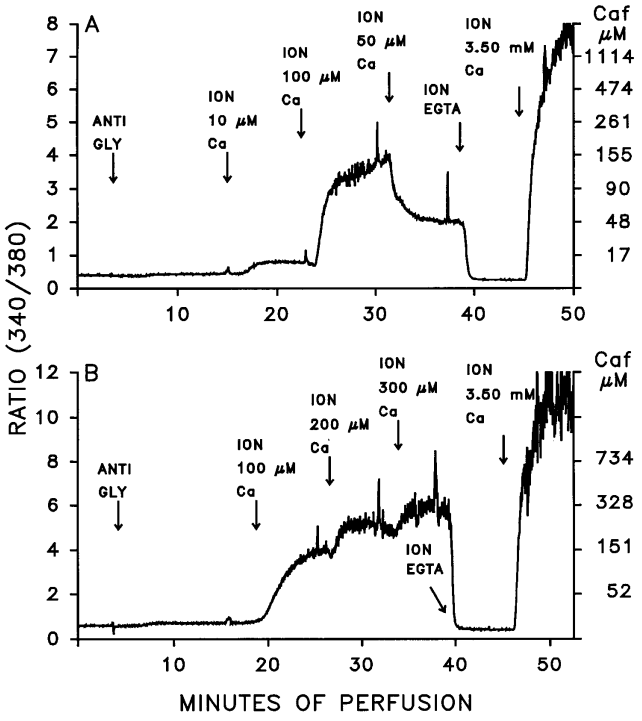

Figure 2. Fluorescence changes in antimycin + ionomycin–permeabilized, mag-fura-2–loaded tubules in media with various defined micromolar Ca^{2+} levels. Tubules were superfused with Ca,Mg-free Solution C for a baseline period followed by introduction of 1 μM antimycin (*ANTI*) + 2 mM glycine, followed by successive exposures to Mg-free Solution C containing 15 μM ionomycin (*ION*) in the continued presence of antimycin + glycine and the indicated Ca^{2+} concentrations. At the end of the experimental period, a calibration procedure consisting of superfusion with Ca,Mg-free Solution C containing 2.5 mM EGTA, followed by superfusion with 15 μM ionomycin in Mg-solution C with 3.50 mM Ca^{2+}, then 200 μM $MnCl_2$ (not shown) was done. All calibration solutions contained 5 mM glycine. The left axis shows the 340/380 ratios, the right axis shows the calculated Ca_f in micromolar using a K_d of 33 μM. (*A*) Experiment with 10, 50, and 100 μM Ca^{2+} medium. (*B*) Experiment with 100, 200, and 300 μM Ca^{2+} medium. Studies are representative of $n = 16$.

glycine, lytic membrane damage, which is recognizable in the superfusion system by rapid parallel decreases in both the 340 and 380 nm signals resulting from accelerated dye leakage (12), did not develop in any of these experiments or the subsequent fluorescence work.

Based on observations such as those shown in Fig. 1, further studies were done in Mg^{2+}-free medium at various levels of Ca^{2+} to better define the Ca^{2+} sensitivity of mag-fura-2 under our experimental conditions. Mag-fura-2–loaded tubules were initially treated with antimycin in the presence of glycine to inhibit ATP-dependent Ca^{2+} pumps that could, particularly at micromolar Ca^{2+} levels, oppose ionophore-induced equilibration of Ca^{2+} between the medium and the intracellular space (20), and then were exposed to ionomycin and varying medium Ca^{2+} levels. As shown in Fig. 2, Ca_f alterations ranging from 10–20 to 300–500 μM were readily discriminated.

Figs. 3 and 4 show representative results of additional studies to further assess the degree to which the presence of Mg^{2+} in the medium affects measurement of fluorescence attributable to changes of intracellular Ca^{2+}. For the experiments in Fig. 3, mag-fura-2-loaded tubules were treated with either ionomycin or antimycin + ionomycin in medium that was either free of added Mg^{2+} or contained 2.4 mM Mg^{2+} with either 100 nM Ca^{2+}, no added Ca^{2+} (Ca-free, which has a contaminating [Ca^{2+}] of 10–15 μM) or 1.25 mM Ca^{2+}. In every case, the responses to ionomycin or antimycin + ionomycin reflected the available Ca^{2+} and were not affected by the Mg^{2+}. In the studies shown in Fig. 4, tubules were initially superfused with Ca,Mg-free medium containing antimycin + ionomycin followed by introduction of either 1.25 mM Ca^{2+} (Fig. 4 *A*), 170 μM Ca^{2+} then 2.4 mM Mg^{2+} (Fig. 4 *B*), or 2.4 mM Mg^{2+} then 170 μM Ca^{2+} (Fig. 4 *C*). Introduction of Mg^{2+} during the experimental periods had little or no effect on fluorescence. Taken together with the observations in Figs. 1 and 2, these data indicate that fluorescence changes observed in mag-fura-loaded tubules under conditions where Ca_f excursions in the micromolar range occur almost entirely reflect these changes in Ca_f with little contribution from intracellular or extracellular Mg^{2+} irrespective of the presence of ATP depleting conditions and/or divalent cation ionophores.

The ATP depletion conditions of interest required observation for up to 60 min during the experimental period as well as time for the baseline control perfusion, recovery when it was studied, and the terminal calibration procedures. Also, just before the calibration procedure to obtain Rmin and Rmax, we interposed a period of perfusion with antimycin + ionomycin + glycine in 10, 50, or 100 μM Ca^{2+} medium to provide an additional point of comparison with the spontaneous changes of Ca_f that developed during ATP depletion. To obtain information from more than a single tubule during these experiments, we performed studies in which repeated measurements were made on the same areas of 7–9 nearby tubules during the sequential phases of the experiment (Fig. 5). These data are shown as the raw 340/380 fluorescence ratios to better illustrate the behavior of the individual tubules in the fashion that the data were collected. For each tubule, represented by a unique symbol, there is a baseline measurement, several measurements during treatment with antimycin + glycine (Fig. 5, *B* and *C*), or a corresponding period of time control observation (Fig. 5 *A*), a measurement in the presence of antimycin + ionomycin + glycine in 10 or 100 μM Ca^{2+} medium, a measurement during determination of Rmin in the presence of ionomycin and EGTA-containing Ca-free medium, and, finally, Rmax in the presence of ionomycin in 3.5 mM Ca^{2+} medium. [Mg^{2+}] in all solutions was 2.4 mM.

Fig. 5 *A* shows a stable baseline signal in the control until introduction of the 100 μM Ca medium with antimycin + ionomycin + glycine. In contrast, as seen in Fig. 5, *B* and *C*, the majority of tubules treated with antimycin + glycine developed increases of the fluorescence ratio beginning after 30 min of exposure. That these values exceeded 10–100 μM Ca^{2+} is shown by the decreases of the fluorescence ratios seen during the subsequent superfusion with ionomycin and 100 or 10 μM Ca^{2+} medium. Calculations of Ca_f in 46 tubules studied during six such experiments are summarized Fig. 6. In 91% of the tubules, Ca_f reached a level greater than 10 μM. In 22% of the tubules, these increases were in the range 10–100 μM, however, the other 68% had increases to greater than 100 μM. In 35% of the tubules, calculated Ca_f exceeded 500 μM, although these values should be considered approximate because of their distance from the K_d. Similar behavior was observed in

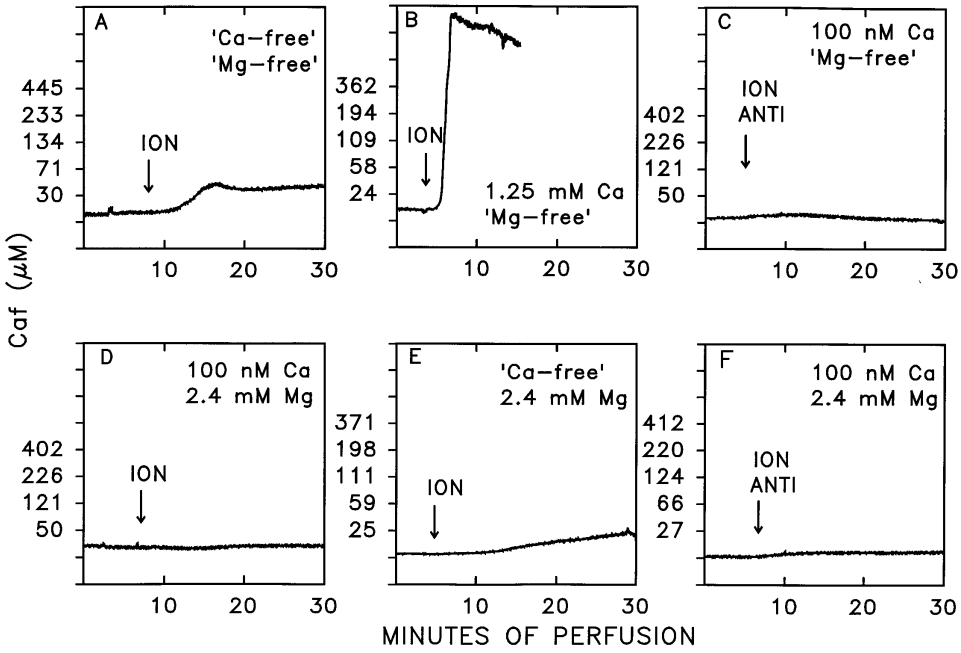

Figure 3. Effects of Mg^{2+} concentration on ionomycin-induced changes in the fluorescence signal at varying medium Ca^{2+} levels. Tubules were superfused in Solution C containing the indicated concentrations of Ca^{2+} and Mg^{2+} in the presence of 2 mM glycine followed by introduction of 15 μM ionomycin (*ION*) (*A*, *B*, *D*, and *E*) or ionomycin plus 1 μM antimycin (*ANTI*) (*C* and *F*). Each of the experiments also had a terminal calibration procedure (not shown) as described in Fig. 2 that was used to obtain Rmin and Rmax for calculation of the Ca_f values shown. Experiments are representative of results from $n = 14$.

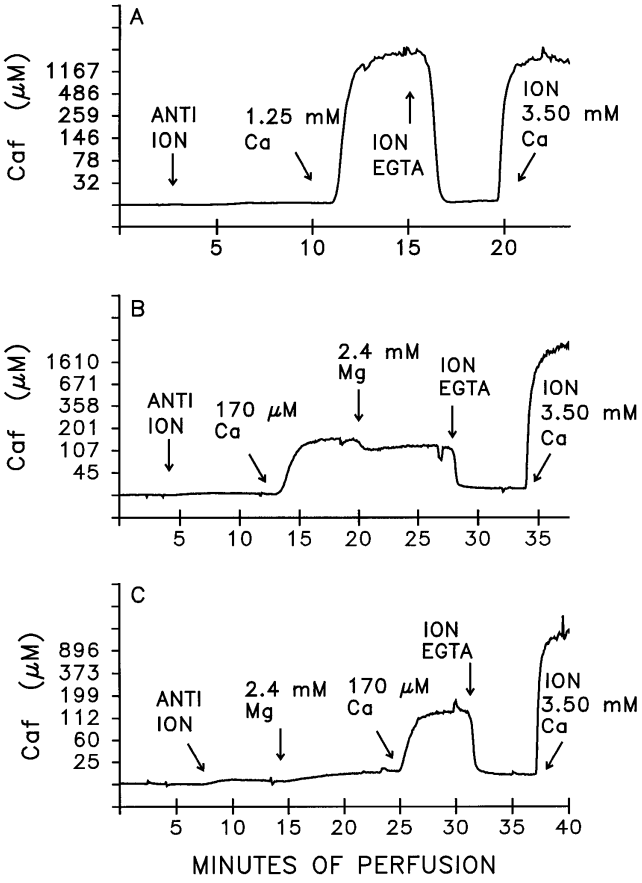

Figure 4. Effect of Mg^{2+} and Ca^{2+} concentration changes during superfusion on Ca_f measurements. Glycine (5 mM) was present during all phases of the experiment. Tubules were initially superfused in Ca,Mg-free Solution C supplemented with 100 μM EGTA, followed by introduction of 1 μM antimycin (*ANTI*) plus 15 μM ionomycin (*ION*) then, in the continued presence of antimycin plus ionomycin, Solution C with either 1.25 mM Ca^{2+} (*A*), 170 μM Ca^{2+} then 2.4 mM Mg^{2+} (*B*) or 2.4 mM Mg^{2+} then 170 μM Ca^{2+} (*C*). Each experiment ended with a terminal calibration procedure consisting of superfusion with ionomycin in Ca-free Solution C containing 2.5 mM EGTA and 2.4 mM Mg^{2+} followed by ionomycin in Solution C containing 3.5 mM Ca^{2+} and 2.4 mM Mg^{2+}, then 200 μM $MnCl_2$ (not shown). Representative of $n = 11$.

six additional experiments in which ATP depletion was induced by the uncoupler, FCCP, instead of antimycin (not shown).

We have mainly used metabolic inhibitors, rather than hypoxia, for our studies of Ca_f in ATP-depleted tubules because of difficulty in reproducibly maintaining O_2 tension in the superfusion system at lower than 10 mm Hg as required for truly hypoxic conditions (42). However, the unexpectedly large magnitude of the Ca_f changes seen with antimycin made an assessment of hypoxia necessary to determine both whether increases of this degree occur during a more natural insult and whether they are reversible. To achieve the necessary degree of hypoxia, we included the oxygen consuming enzyme preparation, Oxyrase, in the medium (37) and all components of the perfusion system ahead of the chamber were kept under N_2/CO_2. Also, to further establish the general applicability of the estimates of Ca_f made with mag-fura-2 and their independence of Mg^{2+}, we used an alternative, newly available, low Ca^{2+} affinity fluorophore, fura-2FF, that is not Mg-sensitive for the hypoxia studies. Fig. 7 *A* illustrates the results of a representative study of this type using the multi-tubule protocol and including both a reoxygenation measurement and an extra internal calibration measurement with 50 μM Ca^{2+} medium in the presence of antimycin + ionomycin + glycine before the usual terminal calibration procedure. The fluorescence ratios increased during hypoxia in all tubules to values that reached or exceeded those measured subsequently in the same tubule

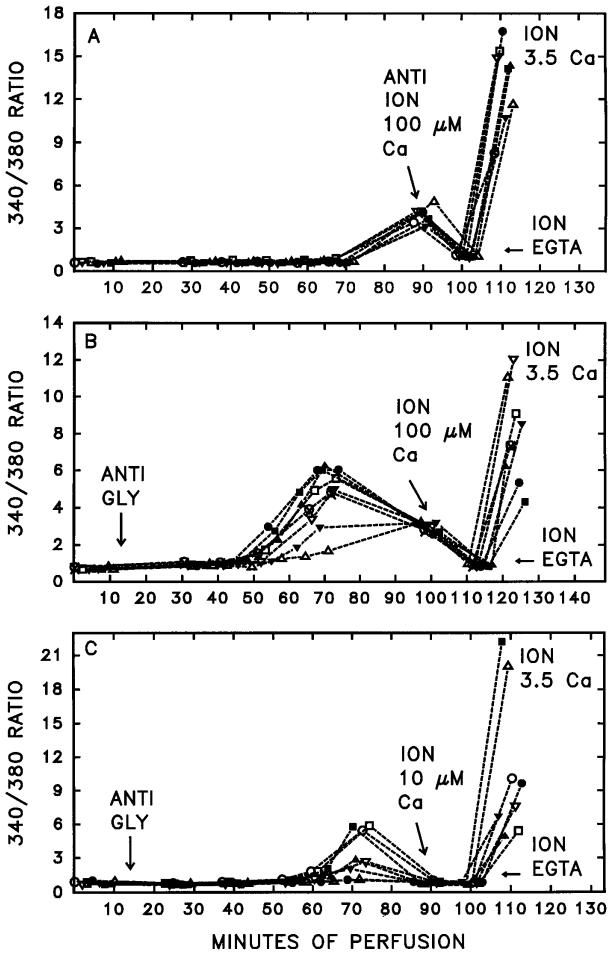

Figure 5. Effects of ATP depletion induced by antimycin in multiple tubule experiments. For each tubule, indicated by a unique symbol, the first time point is the reading at the end of the baseline period of perfusion under control oxygenated conditions. Tubules then received either no further additions (*A*), or, at the points indicated by the arrows, 1 μM antimycin (*ANTI*) plus 5 mM glycine (*GLY*) (*B* and *C*). At the end of the experimental period, just before the calibration steps, an additional superfusion with 1 μM antimycin plus 15 μM ionomycin (*ION*) plus 5 mM glycine in 100 μM (*A* and *B*) or 10 μM (*C*) Ca^{2+} medium was included. The arrows for the ION, 10 or 100 μM Ca labels indicate the points at which the responses to those conditions were measured. The last two points labeled are those used for measurement of Rmin in the presence of ionomycin (*ION*) and EGTA-containing Ca-free medium, and, finally, Rmax in the presence of ionomycin in 3.5 mM Ca^{2+} medium. Mg^{2+} (2.4 mM) was present in all experimental and calibration solutions. Data are shown as 340/380 ratios because the differences in Rmax between the individual tubules preclude a single common scale for the graph based on calculated Ca_f, other than a linear one which would not optimally show each tubule's behavior. The studies shown are representative of $n = 6$ for antimycin-treated tubules and three controls. Six additional studies using the uncoupler, FCCP, instead of antimycin that did not include the full series of calibration steps also gave similar results.

during ionophore permeabilization in 50 μM Ca^{2+} medium and the hypoxia-induced increases of Ca_f recovered during reoxygenation in 6 of the 7 tubules. Fig. 7 *B* illustrates the results of a similar experiment except that the medium Ca^{2+} was kept at 100 nM during hypoxia. This prevented the increases of Ca_f

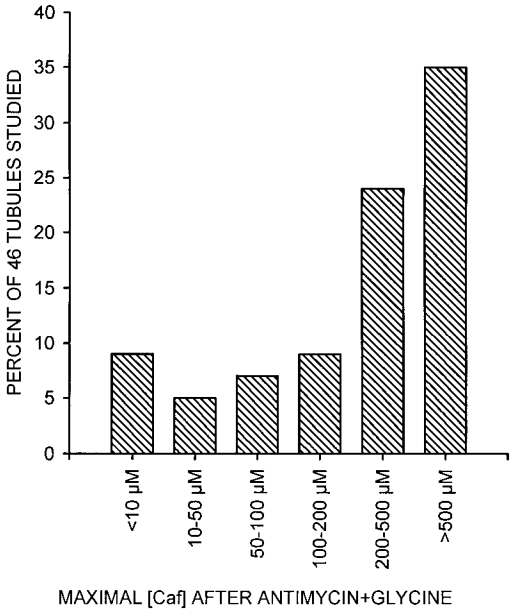

Figure 6. Distribution of calculated Ca_f in multitubule studies with antimycin + glycine. Data are from 46 tubules studied in six separate experiments such as those in Fig. 5, *B* and *C*.

during hypoxia. The fluorescence change induced by 50 μM Ca^{2+} in each tubule is illustrated by the subsequent perfusion with antimycin + ionomycin + glycine in 50 μM Ca^{2+} medium just before the terminal calibration steps.

The levels of Ca_f measured during ATP depletion produced by antimycin or hypoxia are well in excess of those at which mitochondria are considered to buffer increased Ca_f (43, 44). To assess whether a substantial amount of such buffering was occurring in the glycine-protected tubules, we measured total cell Ca^{2+}, increases of which can reflect mitochondrial sequestration under injury conditions (6). Table I summarizes the measurements of total cell Ca^{2+} and of lytic membrane damage as measured by LDH release. During up to 60 min of either hypoxia or antimycin in the presence of glycine, there were no significant changes of total cell Ca^{2+} as compared to controls. As expected, glycine provided virtually complete protection against LDH release (15, 16).

Discussion

It is well-documented that Ca_f is normally kept at ~ 100 nM or less, i.e., 1/10,000th the extracellular concentration, releasable intramitochondrial Ca^{2+} is minimal, an inositol trisphosphate-sensitive Ca^{2+} pool sufficient to increase Ca_f to high nanomolar levels is present in specialized forms of the endoplasmic reticulum, and considerable additional Ca^{2+} can be bound to cellular macromolecules (45–48). Observations consistent with these concepts have been made in kidney proximal tubules using fluorescent intracellular Ca^{2+} indicators (12, 49) and electron probe microanalysis (50–52). Increases of Ca_f are opposed by combined actions of endoplasmic reticulum and plasma membrane Ca-ATPases and plasma membrane Na^+/Ca^{2+} exchange (45, 46, 48), although the latter process may not be expressed in proximal tubules (53). The mitochondrial Ca^{2+} transporter contributes at higher Ca_f than the other mecha-

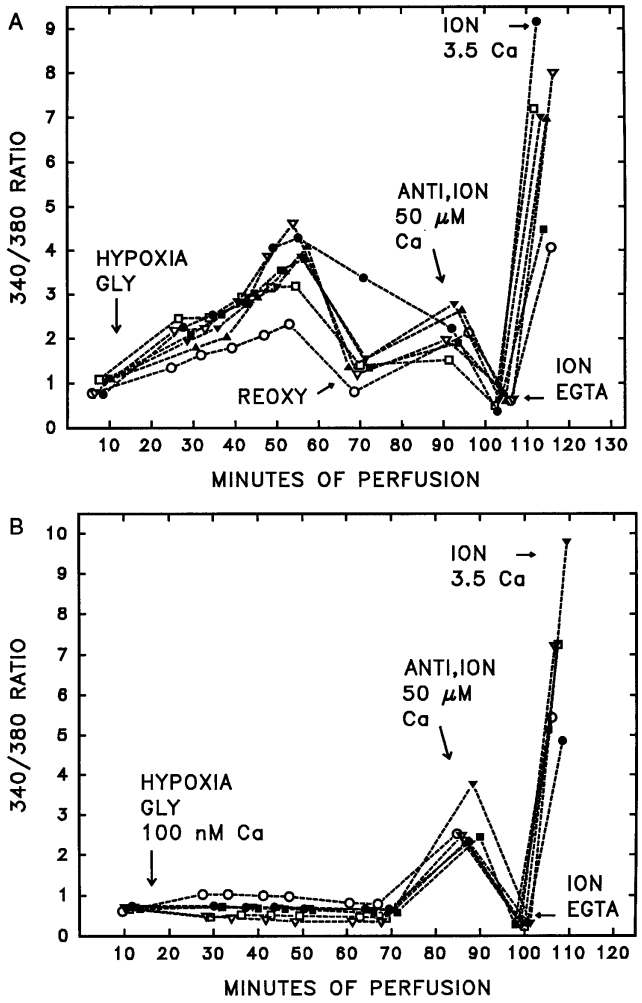

Figure 7. Representative multiple tubule studies illustrating effects of hypoxia in fura-2FF–loaded tubules. (*A*) Hypoxia followed by reoxygenation in normal 1.25 mM Ca^{2+} medium. Results from each of seven tubules are represented by a unique symbol. The first point shown is at the end of the baseline period during superfusion under control, oxygenated conditions. Five mM glycine (*GLY*) was present throughout all experimental maneuvers and the calibration period. Hypoxia in the presence of Oxyrase to maximize the degree of oxygen deprivation was started at the indicated time followed by five readings. Tubules were then reoxygenated in medium that was further supplemented with 4 mM α-ketoglutarate, 4 mM aspartate, and 250 μM MgATP to promote metabolic recovery and an additional reading taken at the point marked REOXY. Then they were superfused with 1 μM antimycin plus 15 μM ionomycin (*ANTI,ION*) in 50 μM Ca^{2+} medium with a reading taken at the indicated point. Labeling of the terminal calibration maneuvers is the same as for Fig. 5. Similar patterns of behavior were seen in all five experiments of this type that were done, each of which assessed 5–7 tubules. (*B*) Similar protocol and labeling as in *A* except the superfusion medium Ca^{2+} concentration was 100 nM and there was no reoxygenation period.

Table I. Total Cell Ca^{2+} and LDH Release in Glycine-protected, ATP-depleted Tubules

		Ca^{2+} (nmol/mg protein)		LDH (percentage free)	
	n	Time control	Experimental	Time control	Experimental
60′ Hypoxia	5	15.6±0.4	12.5±0.8	10.5±1.1	13.3±1.2
60′ Antimycin	5		12.6±0.5		14.4±0.8
30′ Hypoxia	3	15.2±1.2	18.0±2.4	N.D.	N.D.
15′ Antimycin	5	19.4±1.1	18.5±1.5	12.8±0.6	14.0±0.3

Value are means±SEM for the indicated number (*n*) of separate tubule preparations studied. 60′ Hypoxia and 60′ Antimycin experiments had the same time control. Glycine concentrations were: 5 mM for 60 min studies, 4 mM for 30 min studies, and 2 mM for 15 min studies. N.D., not done.

nisms (43, 44). Several factors during injury states potentially favor increases of Ca_f, i.e., opening of normal voltage dependent influx pathways by depolarization, development of pathologically increased plasma membrane permeability, diminished ATP to drive active plasma membrane extrusion and intracellular sequestration, and diminished mitochondrial electron transport to generate ATP and directly promote mitochondrial uptake.

There have been multiple reports describing increases of tubule cell Ca_f measured with fura-2 in various ATP depletion models using both cultured cells (35, 36, 54–58), and, more recently with improvements in methodology, in freshly isolated proximal tubules that retain the normal fully differentiated phenotype of the epithelium (12, 13, 17, 22–24). In the freshly isolated tubule studies, Ca_f has been shown to increase before lytic plasma membrane damage as indicated by loss of ability to exclude vital dyes (12, 21) or accelerated leakage of the fluorophore Ca^{2+} indicator (12). This has been most clearly evident in the presence of glycine, which prevents lytic membrane damage without affecting increases of Ca_f in either ATP-depleted (12, 13, 17, 21, 23) or ionophore-treated cells (12, 13, 15, 17), thus creating conditions where Ca_f remains elevated for relatively prolonged periods in cells that maintain an apparently intact, limiting plasma membrane. Moreover, the timing and progression of various events in the presence of glycine is probably more relevant to that occurring in vivo where intracellular glycine concentrations are high normally and during ischemia (11, 52, 59) and are decreased, but still substantial, during reperfusion (52, 59). The major sustained increases of Ca_f in all studies that have examined the issue have been dependent on the presence of extracellular Ca^{2+} (12, 15, 21, 23, 24) and, when followed for sufficient lengths of time, have reached at least low μM levels that cannot be accurately quantitated with fura-2 (12, 13, 17, 22). The observations with both mag-fura-2 and fura-2FF in this study provides evidence that these increases of Ca_f in a majority of tubule cells reach 100 μM and higher during 30–60 min of ATP depletion in the presence of glycine. Increases of Ca_f of this magnitude during ATP depletion states have not been recognized previously in any cell type before lytic membrane damage.

Reported K_ds of mag-fura-2 for Ca^{2+} have ranged from 17–60 μM under various measurement conditions (29, 31, 32). Our value of 33 μM using in situ calibration is in good agreement. The 1.5 mM K_d of mag-fura-2 for Mg^{2+} (27, 29) makes it suitable for assessment of free intracellular Mg^{2+} so long as Ca^{2+} does not reach μM concentrations (28–30, 33). This has been the case in previous reports using mag-fura-2 during ATP depletion insults, which have employed cultured cell systems where Ca_f did not exceed high nanomolar levels (34–36). In

contrast, mag-fura-2 has served as a useful indicator of the full magnitude of increased Ca_f in several physiological settings in which changes of Mg^{2+} were minimal or absent, but Ca_f increased well into the μM range (28, 30–32). Despite the potential for shifts of Mg^{2+} related to hydrolysis of MgATP or other consequences of ATP depletion under the injury conditions used in the present studies (34–36), the data indicate that the major changes of mag-fura-2 fluorescence measured in the isolated tubules reflected the behavior of intracellular Ca^{2+}. Manipulations of medium Ca^{2+} at μM levels in the presence of ionomycin resulted in large, consistent changes of intracellular mag-fura-2 fluorescence that yielded a K_d value in good agreement with that expected for the interaction between Ca^{2+} and mag-fura-2 and were not influenced substantially by the concomitant presence of Mg^{2+}. The large fraction of mag-fura-2 that was releasable by digitonin is consistent with a predominant cytosolic origin of the signal. Mag-fura-2 can be compartmentalized at intracellular sites of Ca^{2+} sequestration, and signals from these digitonin-insensitive sites have been used to measure Ca^{2+} levels in them (60, 61). However, the strength of these responses is a function of ATP availability, reflecting their requirement for active uptake (60), which is opposite of the behavior observed in our studies. Although the calibration procedure and K_d used gave highly reproducible values when tested with the solutions of defined μM Ca^{2+} levels on antimycin + ionomycin–treated tubules, our conclusions about the magnitude of the Ca_f increases during ATP depletion do not depend solely on these calculations. The studies in Fig. 5 clearly show that subsequent perfusion with calcium ionophore in 100 μM Ca^{2+} medium decreases the fluorescence ratios in individual tubules from the higher values during previous treatment with antimycin alone. Moreover, these changes were seen long after the development of ATP depletion and in the presence of constant medium Mg^{2+}. They can only be explained by increases of Ca_f during the ATP-depleted state that approach and frequently exceed 100 μM. This conclusion is further supported by similar findings in the hypoxia studies that used a different, more natural ATP-depleting insult and an alternative low affinity Ca_f indicator, fura-2FF. There is only one previously published study with this newly available fluorophore (61). We have estimated its K_d to be 13 μM under our experimental conditions, which is less than the 35 μM stated by the manufacturer and less than the K_d of mag-fura-2, but still very suitable for the work. The hypoxia studies also showed that the large increases of Ca_f measured were not simply the result of a late equilibration with medium Ca^{2+} levels in cells that were about to develop lytic damage despite the presence of glycine, since the increases of Ca_f were reversible in a majority of the tubules and lytic membrane damage did not occur even during the further substantial prolongation of the experiments necessary to incorporate both the reoxygenation measurement and the subsequent step in which the response to 50 μM Ca^{2+} in the presence of ionomycin was assessed.

The interpretation of our data is dependent on the effect of ionomycin to equilibrate intracellular and extracellular free Ca^{2+}. There are numerous precedents in the literature for in situ calibration in both cytosol and intracellular compartments using ionomycin and other divalent cation ionophores with respect to both high affinity Ca^{2+}-sensitive fluorophores as well as the low affinity compounds we have used in this manuscript (26, 60–63). That the conditions under which we used ionomycin in the present studies, i.e., a high concentration (15 μM) in the presence of metabolic inhibition induced by concomitant antimycin or immediately antecedent hypoxia, lead to full equilibration with medium Ca^{2+} is shown by our ability for both fluorophores to obtain K_ds using in situ calibration with ionomycin that approximate those measured with the fluorophores in simple salt solutions. If ionomycin (plus antimycin or prior hypoxia) was not fully equilibrating medium and intracellular Ca^{2+}, the K_ds measured by intracellular calibration would have been far lower than those measured in simple salt solutions. There are also multiple other observations in this manuscript and in our previous work using these systems for the past 10 years that allow us to safely conclude that equilibration has taken place: (*a*) The rates of change upon introduction of the ionomycin (plus antimycin or with antecedent hypoxia) are relatively rapid and then cleanly asymptote; (*b*) the terminal calibration procedure used to measure Rmax includes a very high concentration of medium Ca^{2+}, i.e., 3.5 mM; (*c*) as discussed below, in previously published work (13, 15, 16, 20) we have assessed multiple intracellular metabolic effects of increased free Ca^{2+} induced by ionomycin + antimycin including ultrastructure, phospholipase C activity, phospholipase A_2 activity, and actin polymerization, all of which show the expected sensitivity to increased Ca^{2+} in an incremental fashion as medium Ca^{2+} is increased from 10 μM up to 1.25 mM; (*d*) we have also shown (20) how ionomycin alone, even at 15 μM, is not sufficient to fully equilibrate intra- and extracellular Ca^{2+} because of the continued operation of ATP-dependent pumps, but the combination of ionomycin and antimycin as used for the present studies, overcomes this problem.

The contribution of increased Ca_f to injury during ATP depletion has remained incompletely defined despite much work. In the absence of glycine, prevention of increased Ca_f by lowering extracellular Ca^{2+} (12–15, 21, 42) or the use of an intracellular Ca^{2+} chelator (21) has delayed cell killing in some studies (14, 21, 42), but not others (12, 13, 15). In those studies where effects of Ca^{2+} removal were seen they were partial and relatively transient (14, 21, 42). This behavior may reflect counterbalancing beneficial and deleterious effects of the Ca^{2+} manipulations per se (19, 64). In the presence of glycine, cytoprotective actions of the amino acid are dominant, so that cell killing is strongly suppressed irrespective of the behavior of Ca_f (12, 13, 15, 16), however a variety of potentially important Ca^{2+}-dependent prelethal events become amenable to analysis (13, 15, 16, 20).

Recently, we have characterized the Ca^{2+} dependence of phospholipid degradation and actin polymerization during ATP depletion–induced tubule cell injury. In tubules treated with antimycin and the Ca^{2+} ionophore, ionomycin, in the presence of glycine to maximally equilibrate Ca^{2+} across cellular compartments without lytic membrane damage, 2/3 of unsaturated fatty acid accumulation was Ca^{2+}-independent and the Ca^{2+}-dependent component became evident only when the Ca^{2+} concentration exceeded 10 μM. When tubules were treated in the presence of glycine with antimycin alone or subjected to hypoxia, no Ca^{2+}-dependent component was evident (15). Measurements of actin depolymerization in tubules treated with antimycin + ionomycin + glycine indicated a threshold of 10 μM Ca^{2+} for initiation of actin depolymerization, which was accompanied by swelling of microvilli and disruption of their actin cores (20). However, in glycine-treated tubules subjected to antimycin alone, or hypoxia, similar microvillar alterations developed in a Ca^{2+}-independent fashion

without actin depolymerization (16, 20). The current studies indicate that the failure of these Ca^{2+}-dependent effects to occur during the ATP depletion states is not due to insufficient increase of Ca_f. We have also shown that it is not attributable to any direct effect of glycine to suppress these Ca^{2+}-dependent processes (15, 20). Taken together, these observations suggest that the Ca^{2+}-dependent alterations in unesterified fatty acid accumulation and actin depolymerization occur predominantly in cellular compartments that are protected from the fluorophore-detectable increases of Ca_f during ATP depletion. The apical microvilli and mitochondria are two such sites.

The apical microvilli contain the largest pool of F-actin in the proximal tubule cells (16) and their earliest Ca^{2+}-dependent structural changes correlate closely with alterations of actin polymerization and polyphosphoinositide degradation (13, 16). In this regard, electron microprobe studies have shown increased total Ca^{2+} in the microvillar compartment as compared to the cytosol, which may represent a greater binding capacity that could buffer ATP depletion-induced increases of Ca_f (50, 51).

The mitochondrial phospholipase A_2 that is activated prominently by mitochondrial Ca^{2+} transport and buffering during injury states has a Ca^{2+} requirement in the low micromolar range (65, 66), which is similar to the threshold Ca^{2+} level for the Ca^{2+}-dependent increment of fatty acid accumulation in the antimycin + ionomycin–treated tubule cells (15). The absence of a Ca^{2+}-dependent component of fatty acid accumulation during ATP depletion may, thus, reflect the lack of mitochondrial Ca^{2+} uptake. This possibility is supported by the measurements of total cell Ca^{2+} in the glycine-protected cells subjected to ATP depletion by hypoxia or antimycin alone, which did not show the increases that would be expected if the mitochondria were accumulating Ca^{2+} to buffer the increases of Ca_f (6, 67). The absence of mitochondrial Ca^{2+} accumulation is consistent with the nature of the insult. Mitochondrial Ca^{2+} accumulation can be driven by either electron transport or ATP hydrolysis (6, 46). The Ca_f increases in the ATP depleted tubules occurred in a delayed fashion well after maximal ATP depletion, so ATP-driven uptake would not be possible and both the hypoxic conditions and antimycin profoundly suppress electron transport.

Notwithstanding the apparent absence of Ca^{2+}-dependent events in microvilli and mitochondria, the levels of Ca_f reached in the glycine-protected, ATP-depleted tubules are remarkable when considered in the context of the high degree of overall structural preservation and the cohesiveness and normal appearing texture of the cytoplasmic matrix seen in them (10, 13, 16). The findings, however, are consistent with the retention of many structural features in glycine-protected cells even when they are subject to yet greater perturbations of Ca_f resulting from treatment with metabolic inhibitor plus Ca^{2+} ionophore in Ca^{2+}-replete media (13). The freshly isolated tubule system used in this study does not permit assessment of whether the ATP depleted tubules that have sustained such large increases of Ca_f are truly viable over the long term. Recently, however, we have addressed this question using MDCK cells treated with mitochondrial uncoupler and ionomycin in the presence of glycine in media with Ca^{2+} concentrations ranging from 26 nM to 1.25 mM (68). Exposure to concentrations of Ca^{2+} as high as 100 μM for 3 h was compatible with sustained recovery of metabolic and proliferative function when the cells were returned to normal growth medium. At Ca^{2+} levels > 100 μM and durations greater then 3 h, DNA damage became limiting for long term viability. We do not yet know whether such DNA damage is associated with the increases of Ca_f to > 100 μM that occur in the freshly isolated tubules under ATP depletion conditions in the absence of ionophore. Taken together, however, the data in the present studies and the MDCK cell observations provide evidence for an extraordinary degree of resistance to the potentially disruptive effects of increased Ca_f during ATP depletion states so long as glycine is available and suggest that further investigation of the modification of Ca^{2+}-regulated metabolic events as a function of glycine availability during ATP depletion will provide additional insight into critical determinants of cellular survival and structural integrity.

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