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LABORATORY INVESTIGATION

# Acute phosphate depletion and in vitro rat proximal tubule injury: Protection by glycine and acidosis

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**Acute phosphate depletion and in vitro rat proximal tubule injury: Protection by glycine and acidosis.** The effects of phosphate ($PO_4$) removal from Krebs Henseleit buffer on freshly isolated rat proximal tubules (rPT) were assessed by measuring $Ca^{2+}$ uptake (nmol/mg protein), cellular adenosine triphosphate (ATP) (nmol/mg), tissue $K^+$ content (nmol/mg) and lactate dehydrogenase (LDH) as an index of cell integrity. $Ca^{2+}$ uptake increased by 50% in rPT incubated in zero $PO_4$ medium as compared to control (2.6 ± 0.1 vs. 3.9 ± 0.19, $P < 0.001$) and LDH release increased 2.5-fold from 14.2 ± 0.6 to 31.6 ± 1.6%, $P < 0.001$. Neither verapamil (200 μM) nor mepacrine (50 μM) reduced $Ca^{2+}$ uptake or decreased LDH release suggesting that the increased $Ca^{2+}$ uptake was not occurring through potential operated channels and that phospholipase-induced cell injury was not the cause of increased LDH release. Either glycine (2 mM) or extracellular fluid acidosis (pH 7.06), however, significantly diminished rPT injury and $Ca^{2+}$ uptake. Specifically, as compared to the increased LDH released in untreated, $PO_4$-depleted rPT, LDH release was diminished significantly by glycine treatment (31.0 ± 0.9 vs. 15.5 ± 1.6%, $P < 0.001$) or acidosis (30.3 ± 0.04 vs. 19.2 ± 0.9%, $P < 0.01$). $Ca^{2+}$ uptake did not increase in glycine treated tubules (2.6 ± 0.1 vs. 2.8 ± 0.2 nmol/mg, NS) or in the presence of acidosis (2.6 ± 0.1 vs. 2.97 ± 0.17 nmol/mg, NS). ATP concentrations were markedly reduced by $PO_4$ depletion (2.8 ± 0.2 vs. 4.8 ± 0.3 nmol/mg, $P < 0.001$) and remained at low levels during either acidosis or glycine-induced protection. ATP depletion was accompanied by loss of $K^+$ from rPT and this was only modestly attenuated by either glycine or acidosis. Total cell $PO_4$ was not significantly altered, however, perchloric acid (PCA) extractable free $PO_4$ was reduced significantly (33.3 ± 4.5 to 15.9 ± 3.5 nmol/mg, $P < 0.01$). The rPT injury, associated with acute $PO_4$ depletion, may be related to $Ca^{2+}$ uptake since $Ca^{2+}$ uptake and LDH release were both attenuated by glycine administration or acidosis.

Chronic phosphate ($PO_4$) depletion has been shown to afford protection against the progression of chronic renal failure [1]. The mechanism of this effect has been attributed to a diminution in renal parenchymal calcium-phosphorus deposition [2] and an attenuation of nephron hypermetabolism [3]. However, chronic $PO_4$ depletion in vivo has also been shown to actually enhance the severity of ischemic acute renal failure, including the degree of tissue and mitochondrial $Ca^{2+}$ deposition [4]. Chronic $PO_4$ depletion is, however, associated with hypercalcemia and hypercalciuria. These increases in the $Ca^{2+}$ concentration of the urine and plasma that bathe the rat proximal tubule (rPT) in vivo could cause the increased tissue and mitochondrial $Ca^{2+}$ content of ischemic renal tissue. Extracellular $PO_4$ concentration can, however, be decreased in vitro without these secondary events on the extracellular $Ca^{2+}$ concentration bathing the luminal and basolateral membranes. In vitro studies in isolated perfused rabbit proximal tubules have demonstrated that an acute decrease in extracellular $PO_4$ alone diminishes tubular solute and fluid transport and mitochondrial respiration [5, 6].

The present study was undertaken to further evaluate the in vitro effects of acute $PO_4$ depletion alone on rPT function; the results indicate: (1) cellular damage with increased lactate dehydrogenase (LDH) release; (2) increased tubular $Ca^{2+}$ uptake independent of potential activated $Ca^{2+}$ channels and phospholipase activation; and (3) attenuation of membrane damage by glycine and extracellular acidosis.

## Methods

### *Preparation of rPT in suspension*

In each experiment, a single, male Sprague-Dawley rat (225 to 275 g, body wt) was sacrificed in order to harvest rPT. rPT from the renal cortex were harvested using Percoll gradient centrifugation techniques similar to those described by Almeida et al [7] and Gesek, Wolff and Strandhoy [8].

Specifically, following anesthesia with sodium pentobarbital (35 mg/kg, i.p.), the abdomen was opened and a catheter (PE90) inserted into the abdominal aorta. The aorta above and below the renal arteries was ligated and 30 ml of Krebs Henseleit buffer (KHB), warmed to 37°C, gassed with 95% $0_2$/5% $CO_2$ and containing in mM, 118 NaCl, 24 $NaHCO_3$, 4 KCl, 1 $KH_2PO_4$, 1.3 $CaCl_2$, 1.2 $MgCl_2$, 2.5 HEPES plus collagenase (Worthington CLS 1, 40 mg) and hyaluronidase (Type III, 20 mg; Sigma Chemical Co., St. Louis, Missouri, USA), were flushed through the catheter over 10 minutes. The renal veins were severed approximately 15 seconds after beginning perfusion. After perfusion, the kidneys were rapidly removed, placed in ice-cold, gassed KHB and 3 to 5 thin slices were removed from the dorsal and ventral surfaces of each kidney using a Stadie-Riggs microtome, which removes 0.5 mm thick sections. The residual kidney cortex and the slices were diced on a cold petri dish. The diced tissue containing glomeruli, vascular tissue and cortical tubules was washed twice with cold KHB to remove cellular debris and enzymes (50 × g, 30 seconds, refrigerated ICN

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centrifuge). The tissue was then suspended in 30 ml of KHB containing 45 mg of collagenase and placed in a shaking water bath at 37°C for 30 minutes. The suspension was centrifuged and the pellet of tissue was incubated in KHB containing bovine serum albumin (2.5%) for 10 minutes to neutralize the effects of collagenase. The tissue was pressed through a tea strainer and washed three times with cold KHB to remove residual albumin and neutralize any remaining collagenase. The pellet was then suspended in a 45% Percoll solution.

During incubation of the renal tissue in collagenase for 30 minutes, a Percoll (Pharmacia, Upsala, Sweden) solution was prepared as follows: 54 ml of cold Percoll was added to 6 ml of cold, concentrated (10-fold) KHB. This solution, termed 100% Percoll, was gassed with 95% $O_2$/5% $CO_2$ and then the pH was adjusted to 7.4. The 100% Percoll (27 ml) was then added to 33 ml of $Ca^{2+}$-free KHB; the resultant 60 ml (45% Percoll) was gassed for five minutes with 95% $O_2$/5% $CO_2$ and the pH adjusted to 7.4. After washing, the final tissue pellet was suspended in the 60 ml of 45% Percoll and after mixing was divided into two 30 ml aliquots in polypropylene centrifuge tubes. A 5 ml cushion of 100% Percoll was placed at the bottom of each tube which was then centrifuged in a Sorval refrigerated centrifuge at 10,000 rpm (approximately 17,500 × g) for 10 minutes.

rPT, 98% pure as assessed by microscopic examination, were harvested from the lowest of three separated bands of tissue. This tissue was washed twice with cold KHB, suspended in KHB containing 5 mM glucose, 4 mM lactate, 1 mM butyrate, and 1 mM glutamate, and placed in 25 ml Erlenmeyer flasks; 7 ml of rPT suspension were added to each flask and each ml contained approximately 1 mg of protein. These flasks were gassed for five minutes with 95%$O_2$/5% $CO_2$ and then sealed with rubber stoppers and placed in a shaking water bath to warm slowly to 37°C. After 15 minutes, the rPT were gently pelleted and resuspended in fresh, oxygenated media, a procedure designed to remove extracellular enzymes such as proteases which may have leaked from the cells as they progressed from the cold, slightly swollen state to 37°C. Studies were conducted 25 minutes after this procedure. In the case of free $PO_4$ rPT, the final wash was carried out with 30 ml of cold free $PO_4$ KHB as was the subsequent gassing and incubation of 7 ml aliquots in Erlenmeyer flasks. These rPT were exposed to free $PO_4$ medium for 40 minutes.

### *$Ca^{2+}$ uptake and LDH measurements*

$Ca^{2+}$ uptake was measured after 40 minutes of incubation in either the $PO_4$-replete or $PO_4$-deplete KHB. At 40 minutes, the flasks which had been incubating in a well oxygenated environment at 37°C were opened and 1 μl/ml of $^{45}CaCl_2$ (SA 2.0 mCi/ml; New England Nuclear, Boston, Massachusetts, USA) was added to each flask which was gently swirled to ensure mixing. The flasks were sealed and returned to the water bath. One minute later duplicate 1 ml samples were removed, added to 30 ml of ice-cold saline (0.9%) and centrifuged at 3,000 rpm for 30 seconds. The supernate was decanted and the tubes with pelleted rPT were inverted to drain; after the tubule pellet had dried it was lysed with 1 ml distilled $H_2O$. After vortexing the tissue, 500 μl was counted in scintillation fluid and two 25 μl samples were assayed for protein using the Lowry method [9]. Duplicate 25 μl samples were also removed from the original rPT suspension to calculate specific activity.

LDH was measured by the method of Bergmeyer [10] as previously described for our laboratory [11]. One ml samples of rPT were removed just before adding $^{45}CaCl_2$. rPT were pelleted by centrifugation at 50 × g, the supernate was removed and assayed for LDH release. The pellet was lysed and analyzed for LDH separately. The percent released was the supernate LDH divided by the total LDH (pellet plus supernate).

### *Morphology*

One ml samples of rPT were added to 1 ml of 1.2% buffered glutaraldehyde and processed for microscopy using techniques previously published [1].

### *Adenine nucleotide measurements*

rPT suspension (1 ml) was centrifuged and the pellet extracted with 0.56 M perchloric acid (PCA) (1 ml). Two aliquots of 25 μl were taken for protein determination [9] and the acidified suspension was neutralized with 4.0 M $K_2CO_3$ (75 μl). Following centrifugation for 10 minutes at 1500 × g, the supernate was filtered and stored at −70°C until measurement.

A Beckman HPLC model 342 and $^{18}C$ resolve cartridge (5 μM) were used [11]. Buffers A and B contained 0.1 M $K_2HPO_4$ and 0.1 M $K_2HPO_4$ in 25% methanol, respectively. The column was equilibrated for two minutes with 2% buffer B at 1.5 ml/min. The adenine nucleotides were eluted using a 10 minute linear gradient from 2% to 4% buffer B at 1.5 ml/min.

Peaks were quantitated at 254 nm with a Hewlett-Packard model 3390A integrator using the relationship of peak height per picomole concentration of known standards. The results were expressed in nmol/mg protein.

The adenylate energy charge was calculated according to the formula (ATP + 0.5 ADP)/(ATP + ADP + AMP). This value takes into consideration the balance of all the adenine nucleotides and therefore estimates the energy state of the cell [12].

### *Total and free $PO_4$ and $K^+$*

For measurements of cell $K^+$ levels, tubules were rapidly separated from the medium by laying 0.5 ml of tubule suspension into a microcentrifuge tube containing 0.7 ml bromododecane (Sigma Chemicals) on top of 0.2 ml of 275 mm sucrose containing 4% Ficoll (Sigma). The tube was centrifuged at maximal speed for 30 seconds to pellet the tubules in the bottom sucrose layer. $K^+$ levels in this bottom layer were measured by atomic absorption spectroscopy and were factored for pellet protein.

For measurement of tubule total $PO_4$, 1 ml of tubule suspension was sampled and washed twice with 10 ml of free $PO_4$ buffer in order to remove the normal medium. The tubule pellet was stored at 4°C until the measurements. For determination of total $PO_4$, the pellet was degraded and $PO_4$ was determined using the molybdate reagent according to Ames and Dubin [13]. $PO_4$ levels were factored for tubule protein, measured in 1 ml tubule suspension sampled in parallel with the total $PO_4$ sample.

For assessment of tubule inorganic phosphate levels, tubules were rapidly separated for this medium by spinning through oil, as previously mentioned. The bottom layer consisted of 200 μl of ice-cold 10% PCA. Spinning was done at 4°C and all solutions were kept cold in order to prevent hydrolysis of $PO_4$ esters. The

**Table 1.** $Ca^{2+}$ uptake and membrane injury (LDH release) in rPT exposed to free $PO_4$

| | Normal $PO_4$ KHB $N = 19$ | Free $PO_4$ KHB $N = 19$ | −Glu $N = 9$ | −Glu, −Glut, −Buty, −Lact $N = 4$ | +Verapamil (200 μM) $N = 4$ | +Mepacrine (50 μM) $N = 4$ |
|---|---|---|---|---|---|---|
| $Ca^{2+}$ uptake nmol/mg | 2.60 ± 0.10 | 3.90 ± 0.19 | 3.00 ± 0.02 | 3.60 ± 0.15 | 3.10 ± 0.20 | 3.20 ± 0.17 |
| *P* value | | <.001 | <.01 | <.001 | <.01 | <.05 |
| LDH release % | 14.20 ± 0.60 | 31.60 ± 1.63 | 31.10 ± 2.10 | 25.00 ± 0.20 | 25.00 ± 2.20 | 24.00 ± 2.40 |
| *P* value | | <.001 | <.001 | <.001 | <.01 | <.05 |

Abbreviations are: LDH, lactate dehydrogenase; rPT, rat proximal tubules; KHB, Krebs Henseleit buffer; Glu, glucose; Glut, glutamate; Buty, butyrate; Lact, lactate.

*P* values refer to differences between free $PO_4$ studies and normal $PO_4$.

*N* is the number of experiments.

PCA extract was neutralized with 4.0 M $K_2CO_3$ and stored at −70°C until determination of $PO_4$. $PO_4$ was measured in the PCA extract according to Ames and Dubin [13], omitting the degrading step to prevent as much as possible any hydrolysis of esters.

### *Experimental studies*

Several studies were conducted as modification of the above protocol. Alterations were made just before the rPT were placed in the shaking water bath, some 40 minutes prior to sampling. These additions were made to both the $PO_4$-depleted (experimental) and $PO_4$-repleted (control) rPT.

*Substrate deprivation.* In these experiments either glucose alone or glucose, lactate, butyrate, and glutamate were omitted from the KHB in which rPT were incubated at 37°C.

*Spermine.* Ten microliters of a stock solution was added to increase KHB spermine concentration to either 0.2 or 2.0 mM.

*Mepacrine.* Ten microliters of a stock solution was added to increase the final KHB concentration of mepacrine to 50 μM.

*Glycine.* Ten microliters of a stock solution was added to increase the glycine concentration to 2 mM in the 7 ml of rPT suspension.

*Verapamil.* Ten microliters of a stock solution was added to the KHB such that the final concentration of verapamil (Knoll Laboratories) was 200 μM.

*Acidosis.* HCl (0.1 N) was added to reduce KHB pH to 6.7 prior to beginning incubation of rPT at 37°C in the shaking water bath.

### *Statistical analysis*

Comparisons were made using ANOVA followed by Bonferroni's correction. A *P* value of $P < 0.05$ was considered significant.

## Results

### *Effect of acute $PO_4$ depletion to induce rPT injury*

rPT exposed for 40 minutes to zero $PO_4$ media exhibited increased $Ca^{2+}$ uptake and LDH release. In addition, tissue ATP, adenylate energy change, $K^+$ and free $PO_4$ content, all decreased in rPT incubated in a $PO_4$-depleted medium. Specifically, $Ca^{2+}$ uptake was increased 50% over normal levels and LDH release increased by more than 200% (Table 1). Because steady state conditions were probably not present during this metabolic stress, multiple samples were not taken to evaluate $Ca^{2+}$ kinetic fluxes between cellular compartments [14]. In morphological studies: (1) mitochondrial swelling; (2) marked vacuolization in the terminal web area directly below the brush border; and (3) expansion of lateral and basolateral spaces were noted (Fig. 1). Based on this effect of acute $PO_4$ depletion to increase both $Ca^{2+}$ uptake and LDH release, and to decrease the cellular energy state and electrolyte content, several potential protective maneuvers were evaluated.

### *Effect of substrate removal on acute $PO_4$ depletion-induced rPT injury*

When glucose alone or glucose, glutamate, lactate, and butyrate were eliminated from KHB, $Ca^{2+}$ uptake was only slightly reduced and LDH release remained elevated (Table 1).

### *Effect of verapamil on acute $PO_4$ depletion-induced rPT injury*

Because verapamil pretreatment reduces the severity of hypoxic and anoxic injury to rPT and reduces the characteristic elevation in both $Ca^{2+}$ uptake and LDH release that attend $O_2$ deprivation [7], the effect of verapamil (200 μM) on $Ca^{2+}$ uptake and LDH release was evaluated in this model. As demonstrated in Table 1, addition of verapamil did not normalize either LDH release or $Ca^{2+}$ uptake rate.

### *Effect of phospholipase inhibition on acute $PO_4$ depletion-induced rPT injury*

An increased rate of $Ca^{2+}$ uptake may increase cytosolic-free $Ca^{2+}$ thereby activating $Ca^{2+}$-dependent phospholipases, which could induce plasma membrane damage. Mepacrine (50 μM), a phospholipase inhibitor, was therefore added to the rPT incubated in zero $PO_4$ media for 40 minutes. As is shown in Table 1, no protective effect on either $Ca^{2+}$ uptake or LDH release was observed.

### *Effect of glycine on acute $PO_4$ depletion-induced rPT injury*

Glycine addition to zero $PO_4$ media, which did not change extracellular pH, completely attenuated LDH release and blocked the increase in $Ca^{2+}$ uptake in rPT. Figure 2 demonstrates this unique effect of glycine to preserve cell integrity. Treatment with glycine also reduced the severity of mitochondrial swelling and virtually eliminated the vacuoles at the apical surface of the cells (Fig. 1). Glycine treatment did not prevent the reduction in $K^+$ or free $PO_4$ content of rPT exposed to zero $PO_4$ medium (Table 2).

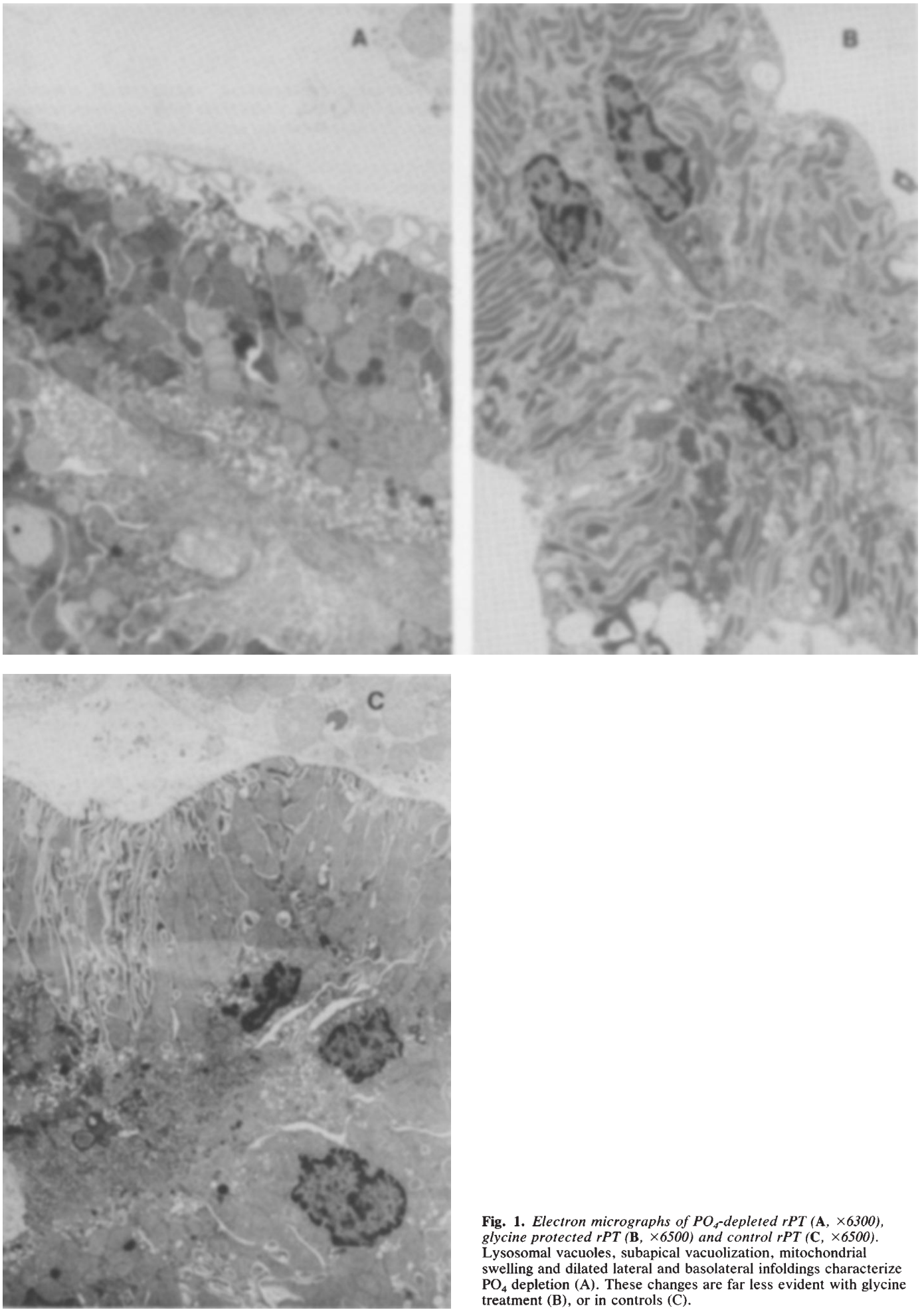


**Fig. 1.** *Electron micrographs of $PO_4$-depleted rPT* (**A**, *×6300*), *glycine protected rPT* (**B**, *×6500*) *and control rPT* (**C**, *×6500*). Lysosomal vacuoles, subapical vacuolization, mitochondrial swelling and dilated lateral and basolateral infoldings characterize $PO_4$ depletion (A). These changes are far less evident with glycine treatment (B), or in controls (C).

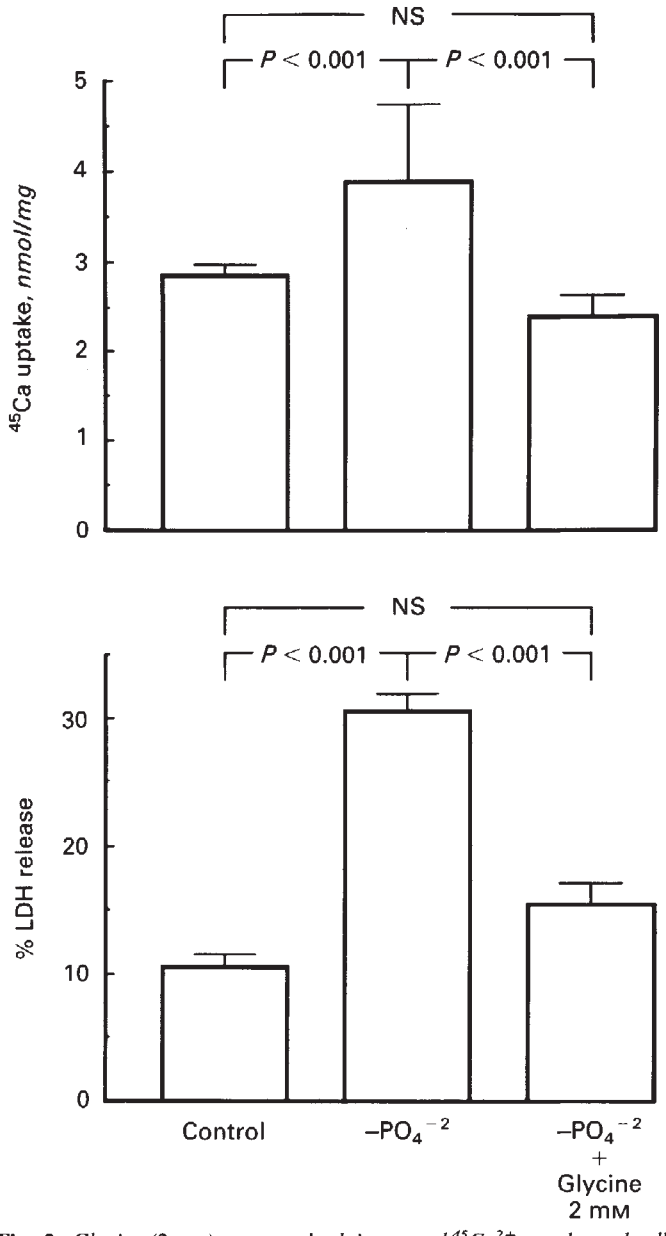


**Fig. 2.** *Glycine (2 mM) prevents both increased $^{45}Ca^{2+}$ uptake and cell injury (LDH release) in $PO_4$-deplete ($-PO_4$) rPT.*

To further explore the role of glycine as a cellular protective agent, spermine (which is synthesized intracellularly in part from glycine), was added to the zero $PO_4$ media at either 0.2 ($N$ = 3) or 2.0 mM ($N$ = 2). Protection against the increased LDH release was not observed as LDH release ranged from 26 to 58%. Therefore, no studies on the effects of spermine on $Ca^{2+}$ uptake were performed.

*Effect of extracellular acidosis on $PO_4$ depletion-induced rPT injury*

Acidosis was achieved by lowering free $PO_4$ KHB pH from 7.4 to 7.0 via addition of HCL. In pilot studies it was found, if pH was lowered to 7.0 in the cold, on warming pH rapidly increased to 7.2 and no protection was observed. pH was therefore reduced in the cold to 6.7 and the final pH on warming averaged 7.06 ± 0.06. Under these latter conditions, acidosis greatly attenuated both the increase in LDH release (30.3 ± 0.04 vs. 19.2 ± 0.9%, $P$ <0.01) and the increase in $Ca^{2+}$ uptake (3.9 ± 0.2 vs. 2.97 ± 0.17 nmol/mg, $P$ < 0.01). This protection occurred without significant changes in either $pO_2$ (478 ± 106 vs. 428 ± 70 mm Hg, $P$ = NS) or $pCO_2$ (28 ± 1 vs. 32 ± 2 mm Hg, $P$ = NS). The protective effects of extracellular acidosis did not involve improvement in $K^+$ or free $PO_4$ (Table 2).

*Cellular protection is independent of ATP levels*

As seen in Table 2, the ATP levels in glycine or acidosis treated rPT were nearly the same as the reduced ATP of unprotected $PO_4$-depleted rPT. The adenylate energy charge (AEC) was also similar in all zero $PO_4$ treated rPT, irrespective of whether acidosis or glycine was used to modulate cell injury. Verapamil treated $PO_4$-depleted rPT also exhibited reduced ATP levels (3.55 ± 0.45 nmol/mg protein) and lower AEC (0.6 ± 0.02) when compared with control ($P$ <0.05).

## Discussion

The present study demonstrates that in short term incubation of suspensions of rPT in free $PO_4$, oxygenated KHB is associated with substantial cellular injury. A two- to three-fold increased efflux of intracellular LDH into the extracellular medium occurred in free $PO_4$ as compared to $PO_4$ containing KHB. A 50% increase in $Ca^{2+}$ uptake also occurred. It is impossible to determine whether the increase in $Ca^{2+}$ uptake represents binding to plasma membranes, entry into the cytosol or both. Loss of $K^+$ from rPT, as occurs in a time dependent manner with hypoxia associated injury [15], is known to increase $Ca^{2+}$ influx through potential operated $Ca^{2+}$ channels [16], and this influx can be blocked with calcium channel blockers [17]. In the present study, $PO_4$ depletion was accompanied by loss of $K^+$. However, with free $PO_4$ suspension medium the $Ca^{2+}$ influx into rPT remained elevated in the presence of the calcium blocker, verapamil, and the cellular damage, as assessed by LDH release, was not attenuated. Thus, in contrast to anoxic and hypoxic injury, acute $PO_4$ depletion does not appear to increase cellular $Ca^{2+}$ influx via potential operated channels. Perhaps the modest decrease in $K^+$ which occurs during $PO_4$ depletion does not induce the same degree of membrane potential changes as does $O_2$ deprivation. These findings in rPT thus extend the results in isolated perfused rabbit proximal tubules in which free $PO_4$ media impaired solute and fluid transport and mitochondrial respiration [5, 6].

A large decrease in cellular free $PO_4$ was observed during incubation in $PO_4$-depleted medium whereas no change in total $PO_4$ could be detected. Because of the important role of free $PO_4$ in phosphorylation reactions, it was possible that a Crabtree effect occurred during $PO_4$ depletion in these studies [18]. The Crabtree effect describes the competition for $PO_4$ by the many cellular processes in which $PO_4$ is critically important. Crabtree noted in tumor cells that glucose phosphorylation took precedence over ATP synthesis if $PO_4$ sources were reduced [19]. To evaluate this possibility, either glucose alone or glucose as well as glutamate, lactate and butyrate were removed from the media in which the rPT were incubated for 40 minutes. In the rPT, however, the injurious effect of free $PO_4$ media did not

**Table 2.** Effects of zero $PO_4$ alone or in the presence of glycine or acidosis on metabolic parameters of injury in rPT

| | Control (1 mM $PO_4$) ($N = 4$) | | | Experimental (0 mM $PO_4$) ($N = 4$) | | |
|---|---|---|---|---|---|---|
| | Basal | +Glycine | +Acidosis | Basal | +Glycine | +Acidosis |
| LDH % released | 14.1 ± 2.3 | 8.5 ± 1.2 | 9.4 ± 1.1 | 26.3 ± 3.9[a] | 7.2 ± 1.0 | 13.4 ± 2.2 |
| ATP *nmol/mg prot* | 4.8 ± 0.3 | 5.2 ± 0.2 | 5.2 ± 0.2 | 2.8 ± 0.2[a] | 2.5 ± 0.1[a] | 3.0 ± 0.2[a] |
| Adenylate energy charge | 0.81 ± 0.01 | 0.79 ± 0.01 | 0.82 ± 0.01 | 0.71 ± 0.01[a] | 0.66 ± 0.01[a] | 0.70 ± 0.01[a] |
| $K^+$ *nmol/mg prot* | 196 ± 20 | 205 ± 11 | 183 ± 21 | 127 ± 9[b] | 151 ± 6 | 156 ± 12 |
| Total $PO_4$ *nmol/mg prot* | 312 ± 12 | 306 ± 15 | 295 ± 32 | 274 ± 13 | 263 ± 27 | 246 ± 25 |
| Free $PO_4$ *nmol/mg prot* | 33.3 ± 4.5 | 24.2 ± 0.1 | 27.2 ± 1.7 | 15.9 ± 3.5[a] | 12.8 ± 1.1[a] | 17.4 ± 0.9[a] |

[a] $P < 0.01$ vs. control
[b] $P < 0.05$ vs. control

appear to be dependent on the Crabtree effect since comparable levels of LDH release and $Ca^{2+}$ influx occurred in the presence and absence of glucose alone, and when all substrates were removed.

The effect of cyclosporine to increase $Ca^{2+}$ influx into smooth muscle vascular [20] and mesangial cells [21] is also not altered by calcium channel blockers. The similar findings in acute $PO_4$ depletion in the present study might therefore suggest that rPT membrane damage accounted for the LDH release and the $Ca^{2+}$ influx. The question then is whether the increased cellular $Ca^{2+}$ influx is merely a consequence of the cell injury or actually contributes to the rPT cell injury. In this regard, the present study examined whether activation of phospholipases, due in part to the increased cellular $Ca^{2+}$ uptake, may be involved in the rPT membrane damage. No support for this possibility was, however, found since the phospholipase inhibitor, mepacrine (which has been shown to attenuate hypoxic injury [22]), altered neither the $Ca^{2+}$ influx nor LDH release associated with acute $PO_4$ depletion.

Free $PO_4$ media diminishes oxidative phosphorylation and thus ATP synthesis in rabbit proximal tubules [6]. Mitochondrial respiratory integrity of $PO_4$-depleted rPT was depressed in the present study as assessed by the adenylate energy charge (Table 2). Furthermore, mitochondrial swelling was also seen in these rPT (Fig. 1). If the increase in cellular $Ca^{2+}$ influx was accompanied by subsequent buffering of $Ca^{2+}$ by mitochondria, even further decreases in cellular ATP would be expected to occur [23]. Indeed, in the present study, low ATP levels were consistently demonstrated. Low tissue levels of ATP would further compromise cell integrity by: (1) decreasing $Ca^{2+}$ uptake by the Ca-ATPase dependent endoplasmic reticulum; (2) decreasing cellular $Ca^{2+}$ efflux by the basolateral Ca-ATPase; (3) diminishing ATP dependent mitochondrial $Ca^{2+}$ uptake; and (4) indirectly altering both $Na^+/H^+$ and $Na^+/Ca^{2+}$ exchange. Thus, a decrease in ATP is accompanied by several events that would disturb $Ca^{2+}$ homeostasis. It is important to note, however, that an in vivo decrease in renal ATP to 50% of normal with fructose or glycerol, respectively, has been shown not to impair renal function or to worsen renal ischemic injury [24].

Glycine or acidosis treatments decreased both LDH efflux and $Ca^{2+}$ uptake and yet no improvements in ATP or adenylate energy charge were observed. Furthermore, verapamil was associated with somewhat higher ATP concentrations as compared to glycine or acidosis but no protection, as assessed by LDH release, was afforded by this calcium channel blocker. The maintenance of low ATP levels in glycine protected rPT is in accordance with the observations of Weinberg et al in hypoxic rabbit proximal tubules. These authors demonstrated that less injury occurred if glycine was added to the buffer media; this protection also did not improve ATP levels during hypoxia [25]. Garza-Quintero et al in anoxic rabbit proximal tubules [26] and Baines, Shah and Ho in the isolated perfused kidney model [27] also noted that alanine and glycine prevented functional deterioration and the release of cellular enzymes, such as LDH, without increasing cortical ATP or adenylate energy charge. Finally, glycine also protects rabbit proximal tubules from injury due to ionomycin and yet $Ca^{2+}$ entry into the cytosol is not reduced [28]. These data, taken together, suggest that glycine also acts a step distal to any decrease in ATP and/or increases in cytosolic $Ca^{2+}$.

The demonstrated effect of both glycine or acidosis to protect against the increased cellular $Ca^{2+}$ influx and LDH efflux, which characterize the injury induced by free $PO_4$ media in the rPT, is provocative. Glycine has been shown to decrease rPT injury in several models, including that induced by cisplatinum [29], anoxia [30], and ouabain [31]. The protective effect of glycine in these studies is independent of the $O_2$ radical scavenger, glutathione. Glycine also did not afford protection against the rPT injury induced by free $PO_4$ media by providing substrate for spermine biosynthesis, an agent which has been reported to enhance membrane stabilization [23], because spermine alone did not protect the rPT.

Finally, acidosis reduces $Ca^{2+}$ uptake and/or cellular $Ca^{2+}$ overload in normal renal tissue [32], in hypoxic renal tissue [33, 34], and during reperfusion of hypoxic renal tissue [11], and lessens the severity of cellular injury as assessed by LDH release [11]. Reports [35, 36] that glycine may enter cells with protons suggest that the similar degree of cellular protection with acidosis or glycine treatment in the present study may have a common basis by causing intracellular acidosis. Further studies, however, will be necessary to examine this possibility.

In summary, in vitro $PO_4$ depletion induces rPT membrane injury, which is associated with increased $Ca^{2+}$ uptake and LDH release. This effect is not altered by verapamil, mepacrine or spermine availability. Protection against the injurious effect of $PO_4$ depletion on rPT was observed with glycine or acidosis. In both circumstances the protection was associated with decreased $Ca^{2+}$ uptake and diminished LDH release. Further studies will be necessary to delineate the pathogenesis of the rPT injury as well as the mechanisms of protective maneuvers, that is, glycine and acidosis.

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