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U. M. Rose · S. L. Abrahamse · J. W. C. M. Jansen
R. J. M. Bindels · C. H. van Os

Cellular acidification occurs during anoxia in cultured, but not in freshly isolated, rabbit proximal tubular cells

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Abstract In a variety of cells it has been shown that acidosis is protective against anoxic injury. We have demonstrated previously that proximal tubule (PT) cells in primary culture were more resistant to anoxia-induced cell injury than were freshly isolated cells. Therefore, we asked the question of whether a difference in cellular acidification during anoxia could explain this difference in susceptibility to anoxia. To answer this question, intracellular pH (pH_i) was measured during anoxic incubation of PT cells in culture and those that were freshly isolated. PT cells were incubated in an anoxic chamber at 37°C after loading with 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) or fura-2 acetoxymethyl ester (fura-2-AM). pH_i and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) were measured by digital imaging fluorescence microscopy. During anoxia, pH_i in cultured PT cells decreased from 7.3 ± 0.1 to 6.8 ± 0.1, whereas pH_i in freshly isolated cells did not decrease significantly. In addition, the intrinsic buffering capacities (β_i) in cultured and freshly isolated PT cells were determined and turned out to be the same at a pH_i greater than or equal to 7.3. Below pH_i 7.3, β_i increased several fold in freshly isolated PT cells, and rose to significantly higher levels than in cultured PT cells. During 1 h of anoxia, cell viability of freshly isolated PT cells decreased significantly to 54% ± 2% ($P < 0.05$), while no loss in viability was observed in cultured PT cells. Clamping the pH_i during anoxia at 6.7 and 6.1 significantly increased cell viability in freshly isolated PT cells to 76% ± 5% and 72% ± 4%, respectively ($P < 0.05$). In contrast, prevention of acidification in cultured PT cells during anoxia did not lead to increased cell death. Therefore, the differences in susceptibility to anoxic injury between cultured and freshly isolated PT cells cannot be explained by cellular acidification in cultured cells, but must be sought elsewhere.

Key words Ischaemia · Intracellular pH · Proximal tubule · Primary culture

U. M. Rose · S. L. Abrahamse · R.J.M. Bindels
C. H. van Os (✉)
162 Cell Physiology, University of Nijmegen, Trigon Bldg, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

J. W. C. M. Jansen
Department of Vascular Pharmacology, Solvay Duphar BV, P.O. Box 900, NL-1 380 DA Weesp, The Netherlands

Introduction

ATP depletion and acidosis are prominent features during hypoxic or ischaemic insults in many tissues, including the kidney [28]. In general, ATP depletion results in the disturbance of intracellular ion homeostasis which leads eventually to cell damage [15, 27]. Increased cytosolic Ca^{2+} ($[Ca^{2+}]_i$) has also been suggested to mediate injury during ATP depletion in several organ systems, including the kidney [7, 23, 27, 28], liver [4, 22], brain [2] and heart [14]. It has been shown that acidification of hypoxic tissue, resulting from glycolytic lactate production, ATP hydrolysis and CO_2 accumulation, can enhance the resistance to the damaging effects of O_2 deprivation in the kidney [7, 24, 26–29], cardiomyocytes [3, 16], and hepatocytes [10, 11, 13, 18]. However, the mechanism behind the protection offered by lowering the pH is unknown. In some cells, such as cardiomyocytes, acidosis has an energy-conserving effect caused by a lower energy demand [16]. In addition, intracellular pH (pH_i) plays a role in the preservation of ionic gradients across the plasma membrane during ATP depletion, by decreasing plasma membrane conductance pathways of, for example Ca^{2+}, or inhibition of Ca^{2+}-calmodulin-regulated processes [26]. Moreover, the mechanisms responsible for membrane and cell damage, such as

phospholipid and protein degradation by phospholipases and proteases, appear to be pH dependent, with maximal activity at or near physiological pH and minimal activity at acidotic pH [13]. Protection by extracellular acidosis has been shown to be mediated by intracellular acidification in hepatocytes [6], but until now this has not been confirmed in renal cells [7, 24, 26–29].

Previous studies on anoxia-induced cell injury in proximal tubular (PT) cells revealed a striking difference in the sensitivity to anoxia between freshly isolated and cultured PT cells [20, 21]. The fact that cultured cells were more resistant to anoxia could be due to the presence of a protective factor such as, for example, intracellular acidosis. The objective of the present study was to measure pH_i and $[Ca^{2+}]_i$ during anoxia in cultured and freshly isolated PT cells and to investigate whether cytosolic acidosis plays a role in protection against anoxia-induced cell injury in PT cells.

Materials and methods

Isolation of PT cells

Rabbit kidney PT cells were isolated by immunodissection as described previously [20]. Briefly, kidneys were excised from New Zealand white rabbits (approx. 0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 min on ice with monoclonal antibodies 85C8 and 101E12, recognizing cell surface antigens specific for the PT. After three washes, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 min at 20 °C. The dishes were washed carefully and adherent PT cells were collected and resuspended in a mixture of Dulbecco's Modified Eagles medium (Imperial No. 1 466 14, Hampshire, UK) : Ham's F12 medium (Gibco, No. 041 01765M, Paisley, UK) (1: 1), supplemented with gentamycin (10 µg/ml), $NaHCO_3$ (25 mM), glutamine (14 mM), insulin (5 µg/ml), transferrin (5 µg/ml) hydrocortisone (50 nM), 0.5% (v/v) non-essential amino acids (Gibco, No. 043 01140H), prostaglandin E_1 (70 ng/ml), triiodothyronine (5 pM), Na_2SeO_3 (50 nM), pH 7.4; hereafter this medium is referred to as K_1 medium. PT cells were either used directly or having been cultured. To obtain a primary culture, PT cells were seeded on collagen-coated coverslips (diameter 22 mm; Menzel, Germany) at a density of 2×10^5 cells/cm^2. Cells were grown to confluency in K_1 medium, supplemented with 5% (v/v) fetal calf serum (FCS) during the first 24 h of culture. For experiments with cultured PT cells, the PT cells were used 5–6 days after seeding. In separate experiments, freshly isolated PT cell clumps containing 3–6 cells, were resuspended at a density of 1×10^6 cell/ml K_1 plus 5% (v/v) FCS, and 5-ml aliquots were incubated at 37 °C in 25-cm^2 tissue culture flasks (Costar 3055, Cambridge, (Mass., USA) [21]. Both cultured and freshly isolated PT cells were kept at 37 °C in a humidified incubator under 5% CO_2 in air until use.

Fura-2 and BCECF loading of PT cells

Freshly isolated PT cells were attached to Cell-Tak-coated (Collaborative Research Incorporated, Bedford, Mass., USA) round coverslips (diameter 22 mm; Menzel) as follows: 5 µl pure Cell-Tak on a glass coverslip was dried in air for 1 h at 20 °C, and 100 µl of a PT cell suspension was applied to the Cell-Tak-coated coverslip within 30 min at 37 °C. Cultured PT cells or attached freshly isolated PT cells were loaded with fura-2 by incubating the coverslip with PT cells for 1 h at 37 °C in K_1 plus 5% FCS medium containing 5 µM and 10 µM fura-2 acetoxymethyl ester (Fura-2-AM; Molecular Probes, Eugene, Ore., USA) for cultured and freshly isolated PT cells respectively, 0.02% (w/v) pluronic F127 (Moleuclar Probes), 4% (v/v) FCS and 3 mM probenecid (Sigma, St. Louis, Mo., USA). PT cells were loaded with 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes) by incubating the coverslips for 45 min at 37 °C in K_1 plus 5% FCS medium containing 2 µM BCECF-AM and 3 mM probenecid (Sigma). Thereafter PT cells were incubated for 30 min in K_1 plus 5% FCS at 37 °C for de-esterification. After loading and de-esterification, the PT cells were washed twice in the experimental medium and were used immediately. All experiments were performed in the presence of 0.3 mM probenecid to inhibit fura-2 or BCECF leakage.

$[Ca^{2+}]_i$ and pH_i measurements

Fura-2 flourescence was monitored using digital imaging equipment (MagiCal, Applied Imaging systems, Tyne and Wear, UK). The fura-2-loaded PT cells were alternately excited at 340 nm and 380 nm and the emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS software (Applied Imaging International, Tyne and Wear, UK). The 340–380 nm capturing sequence was interrupted by 30-s periods, of which 10 s was used for cell focusing (at 340 nm excitation) and for the remaining 20 s, the shutter was closed, to avoid bleaching. The MagiCal system has been described in detail by Neylon et al. [17]. $[Ca^{2+}]_i$ was calculated according to the formula derived by Grynkiewicz et al. [12] : $[Ca^{2+}]_i = K_D \times R_{bf} \times [(R - R_{min}) / (R_{max} - R)]$, where K_D (224 nm) is the dissociation constant of fura-2 for Ca^{2+}; R is the ratio of flourescence of the cell at 340 nm to that at 380 nm; R_{max} and R_{min} are the maximal and minimal values of R, respectively, obtained by treating the PT cells with 5 µM ionomycin in the presence and absence [obtained by addition of 2 mM ethyleneglycol bis(ß-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA)] of extracellular Ca^{2+}, respectively; R_{bf} is the maximal 380-nm signal divided by the minimal 380-nm signal.

BCECF fluorescence was measured by using a Newcastle Photonic system (NPS, Newcastle, UK) in which a photomultiplier is connected to a Nikon Diaphot inverted microscope. The BCECF-loaded PT cells were alternately excited at 490 nm and 440 nm and emitted light was collected at 1-s intervals at 520 nm. BCECF ratios were converted to pH_i by clamping pH_i in a high-K^+ medium, containing (in mM) : 112 potassium gluconate, 28 KCl, 10 NaCl, 1 $MgCl_2$, 0.01 $CaCl_2$, 5 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 10 D-glucose, 20 mannitol and 0.01 nigericin. The medium pH was varied between 6.0 and 7.9 by addition of HCl or tris(hydroxtmethyl) aminomethane (TRIS). Individual calibrations were pooled and a mean calibration curve was calculated by means of linear regression.

Intrinsic buffering capacity of PT cells

The intrinsic buffering capacity (β_i) was estimated using a method described by Boyarski et al. [5]. Briefly, all mechanisms for pH_i regulation in the PT cells were inhibited by omitting Na^+ and HCO_3 from the perfusate [composition in mM: 140 *N*-methyl D-glucamine, chloride salt (NMGCl), 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 L-alanine, 10 D-glucose, pH 7.4]. After 6 min, the PT cells were exposed for 4 min to the same solution except that now 40 mM NMGCl was substituted by 40 mM NH_4Cl. Subsequently, the NH_4Cl concentration was stepwise lowered to 20, 10, 5 and 0 mM at 4-min intervals. Each experiment was followed by a pH_i calibration

according to the procedure described above. β_i was calculated from the pH_i change induced by reducing the NH_4Cl concentration according to the formula $\beta_i = (\Delta B/\Delta pH) = -(\Delta A/\Delta pH)$, where ΔB is the amount (in mM) of strong base added to the solution, ΔA is the amount of strong acid, and ΔpH is the resultant change in pH [5].

Anoxic chamber experiment

Anoxic conditions were realized in an anoxic chamber as described previously [20]. Fura-2- or BCECF-loaded PT cells on coverslips were mounted in this anoxic chamber at 37°C. After filling the anoxic chamber with 100% N_2 gassed modified Krebs Henseleit Buffer [KHB; composition in mM: 138 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 L-alanine, 5 L-lactate, 20 HEPES/TRIS and 360 mU/ml Oxyrase (Oxyrase, Ashland, Ohio, USA)], $[Ca^{2+}]_i$ and pH_i were monitored for 45–60 min.

Estimation of cell viability

PT cells were mounted in the anoxic chamber under continuous monitoring of partial O_2 pressure using a Clark-type electrode [20]. After anoxic incubations, cell viability was estimated by means of lactate dehydrogenase (LDH) leakage for cultured cells [20] and by means of trypan blue exclusion of propidium iodide staining for freshly isolated cells [21]. For LDH activity measurements, medium and cells were collected separately. To this end, cells were scraped off the coverslip in 1 ml MilliQ water and the resulting cell suspension was sonicated for 30 s at 100 W to release all LDH. Both cell and medium samples were centrifugated for 5 min at 200 *g* and LDH content was measured as described previously [20]. For trypan blue and/or propidium iodide staining, coverslips with PT cells were incubated for 1–2 min in 0.08% (w/v) trypan blue or 5 µg/ml propidium iodide. The percentage of stained cells was determined by counting using light miocroscopy for trypan blue staining, or by using fluorescence microscopy for propidium iodide staining as described previously [21].

Materials

All chemicals were of the purest grade and obtained from Sigma unless otherwise indicated.

Statistical analysis

All reported data are expressed as means ± SEM. Statistical analysis was performed on ratio and viability values using analysis of variance ($P < 0.05$ being significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher [25].

Results

$[Ca^{2+}]_i$ and pH_i during anoxia

$[Ca^{2+}]_i$ and pH_i were monitored during substrate-free anoxia in cultured and freshly isolated PT cells. Figure 1A shows a typical example of cultured PT cells, where anoxia induced increases in $[Ca^{2+}]_i$ within 20 min, reaching a maximal level within 30 min. After reintroducing O_2 and glucose, i.e. reperfusion, $[Ca^{2+}]_i$

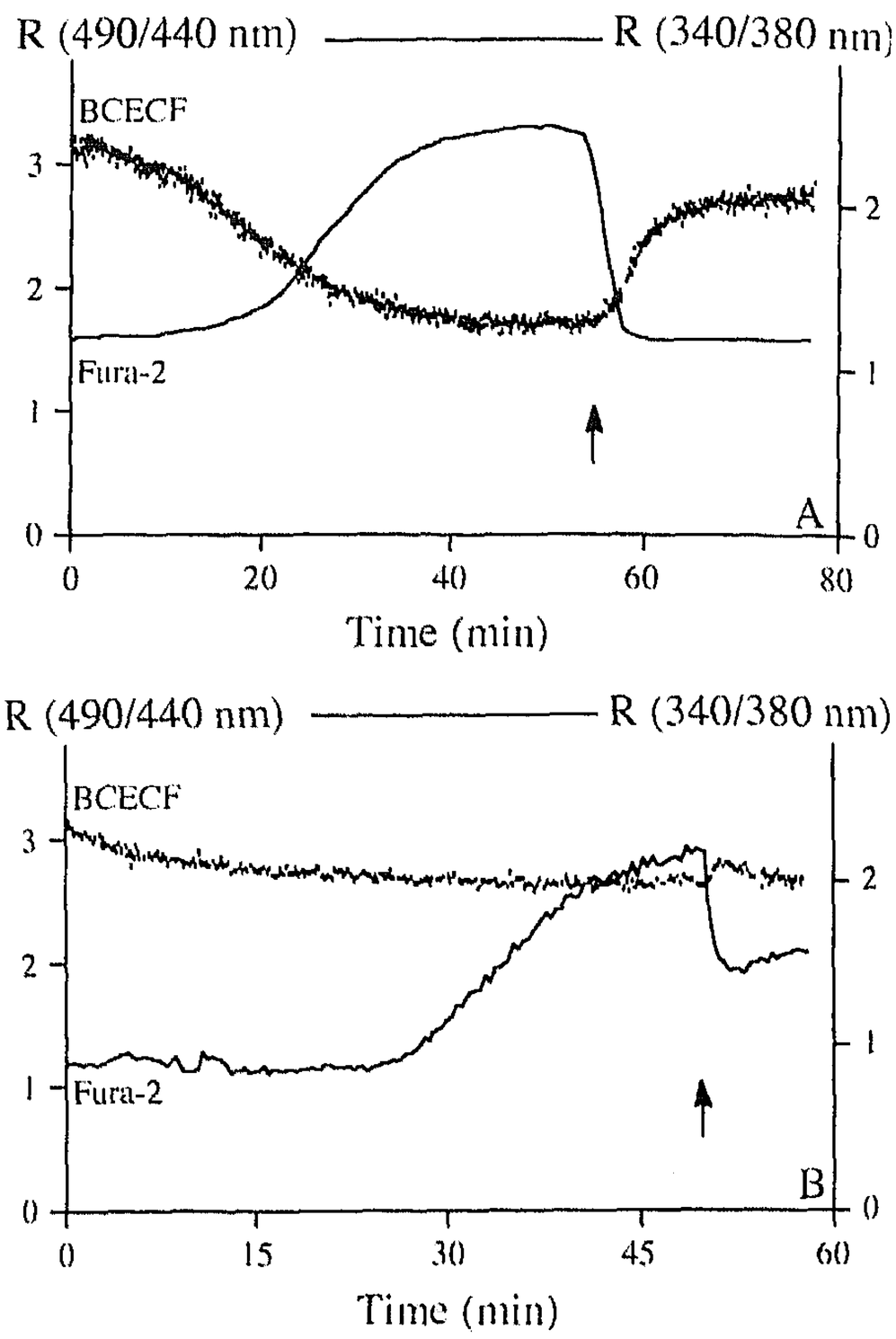

Fig. 1 Typical $[Ca^{2+}]_i$ and pH_i responses of cultured (**A**) and freshly isolated (**B**) PT cells during substrate-free anoxic incubation. Anoxia induced $[Ca^{2+}]_i$ increases in both cultured and freshly isolated PT cells. However, pH_i only decreased in cultured PT cells. $[Ca^{2+}]_i$ is presented as the fluorescence ratio of fura-2 at 340 nm excitation to that at 380 nm, and pH_i as the flouresecence ratio of BCECF at 490 nm excitation to that at 440 nm

returned completely to basal levels, as reported previously [20]. In cultured PT cells, anoxia also induced a decrease in pH_i. The ratio of fluorescence at 490 nm and 440 nm declined significantly from 3.11 ± 0.10 to 2.44 ± 0.11 ($P < 0.05$) within the first 15 min of anoxia. This level was maintained throughout the entire anoxic period until reperfusion. At reperfusion, the BCECF ratio value increased significantly to 3.04 ± 0.11 ($P < 0.05$). Figure 1B shows a typical experiment in which anoxia induced an increase in $[Ca^{2+}]_i$ in freshly isolated PT cells. Upon reperfusion $[Ca^{2+}]_i$ did not always return to initial levels, as described before (Table 1 and [21]). In contrast to cultured PT cells, pH_i did not decrease significantly during 45 min of anoxia in freshly isolated PT cells. In order to provide real pH_i values, a calibration was performed in both cultured and freshly isolated PT cells. PT cells were incubated in a high-K^+ medium containing 10 µM nigericin with a pH ranging from pH 6.0 to pH 7.9. Cultured PT cells were calibrated directly after the anoxic experiment. In some freshly isolated PT cells this was not feasible

Table 1 The effect of intracellular acidosis on $[Ca^{2+}]_i$ in freshly isolated proximal tubular (PT) cells. Anoxic incubation of freshly isolated PT cells at normal and low pH medium. Initial $[Ca^{2+}]_i$ which is $[Ca^{2+}]_i$ 10 min after filling the chamber, anoxic $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ 10 min after reperfusion, are presented in nM. All data are mean values ± SEM of at least 5 experiments. ($pH_o = 7.4$).

Conditions	$[Ca^{2+}]_i$ (nM)	
	$pH_o = 7.4$	$pH_o = 6.0$
Initial	111 ± 16	150 ± 1**
Anoxia	512 ± 33*	412 ± 5*,**
Reperfusion	184 ± 14*	213 ± 7*

*$P < 0.05$ Anoxia and reperfusion values versus pre-anoxic values, **$P < 0.01$ for values at $pH_o = 6.0$ versus $pH_o = 7.4$

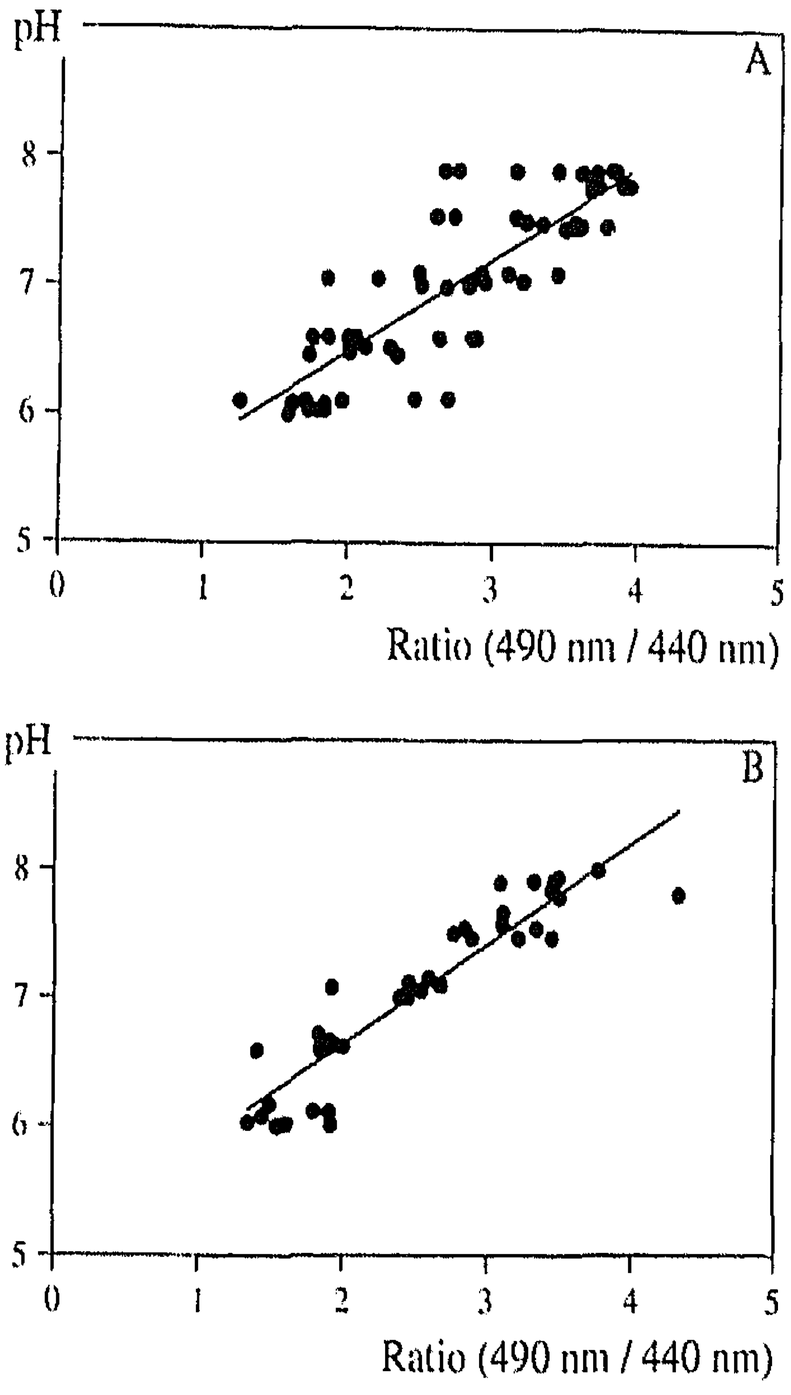

Fig. 2 Calibration curves of pH for cultured (A) and freshly isolated (B) PT cells. PT cells were incubated in high-K^+ buffer containing 10 μM nigericin with pH ranging from 6.0 to 7.9. The calibration curves for cultured and freshly isolated PT cells are $pH = 4.831 + (0.763R)$ and $pH = 5.096 + (0.775R)$, respectively, where pH is that of the buffer and R is the fluorescence ratio at 490 n excitation to that at 440 nm. The correlation coefficients are 0.93 and 0.92 for cultured and freshly isolated PT cells, respectively

since anoxia had induced cell injury and all BCECF fluorescence was lost into the medium. In these cases pH calibrations were performed in separate experiments. Figure 2 shows pH calibration curves for cultured (Fig. 2A) and freshly isolated (Fig. 2B) PT cells. Both curves were linear in the pH range used. From these calibration curves, pH_i values were calculated for

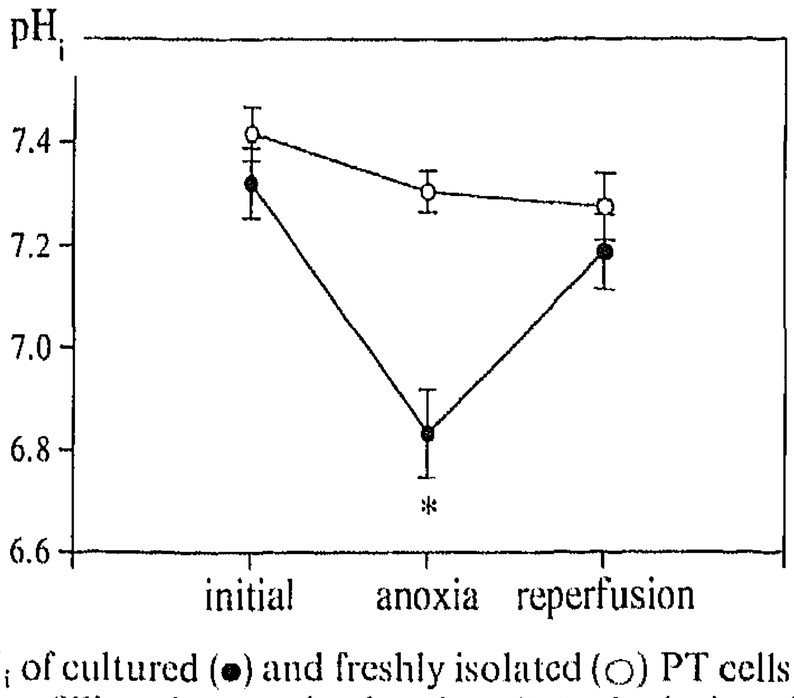

Fig. 3 pH_i of cultured (●) and freshly isolated (○) PT cells measured 10 min after filling the anoxic chamber, (*initial*), during the steady-state reached after 20 min of anoxia (*anoxic*) and at the introduction of O_2 and substrate (*reperfusion*). Each point represents the mean $pH_i \pm SE = M$ with $N \geqq 8$ (*$P < 0.05$ anoxic versus basal pH_i)

cultured and freshly isolated PT cells. Figure 3 gives the pH_i changes which occurred during anoxia and subsequent reperfusion. In cultured PT cells, pH_i decreased significantly from pH 7.3 ± 0.1 to pH 6.8 ± 0.1 during anoxia ($P < 0.05$) and increased again significantly to pH 7.2 ± 0.1 ($P < 0.05$) upon reperfusion, while in freshly isolated PT cells, pH_i did not change significantly ($P > 0.1$).

Protection by cytosolic acidosis

Cell viability of cultured PT cells after 60 min of anoxic incubation was unaltered, as reported previously [20]. Cell viability of freshly isolated PT cells had decreased already during the preparation and preservation of the cells. After the cell isolation procedure, which takes almost 3 h, cell viability was, on average, 84% ± 1%. During the following 5 h of preservation in K_1 medium, cell viability decreased further to 67% ± 3%. We have shown in a previous study that 1 h of anoxia decreased percentage cell viability independent of the time which had passed since the commencement of cell isolation [21]. Therefore, in all experiments, the viability of the control normoxic group was set at 100 % [21]. When expressed in this way, 1 h of anoxic incubation of freshly isolated PT cells decreased cell viability to 54% ± 2%. To test whether intracellular acidosis protects against anoxic injury, pH_i was clamped at low extracellular pH (pH_o) during 60 min of anoxia. To this end, freshly isolated PT cells were incubated in substrate-free high-K^+ medium of pH 6.6 or 6.0 containing 10 μM nigericin. Figure 4 shows a typical experiment of clamping the pH_i at 6.0. During anoxic incubation, the BCECF ratio was stabilized at 1.23, which corresponds to a pH_i of 6.0. On average, pH_i was 6.7 ± 0.1 and 6.1 ± 0.1 during clamping in pH_o 6.6 and 6.0, respectively. After 60 min of anoxic incubation at pH_o 6.6 or

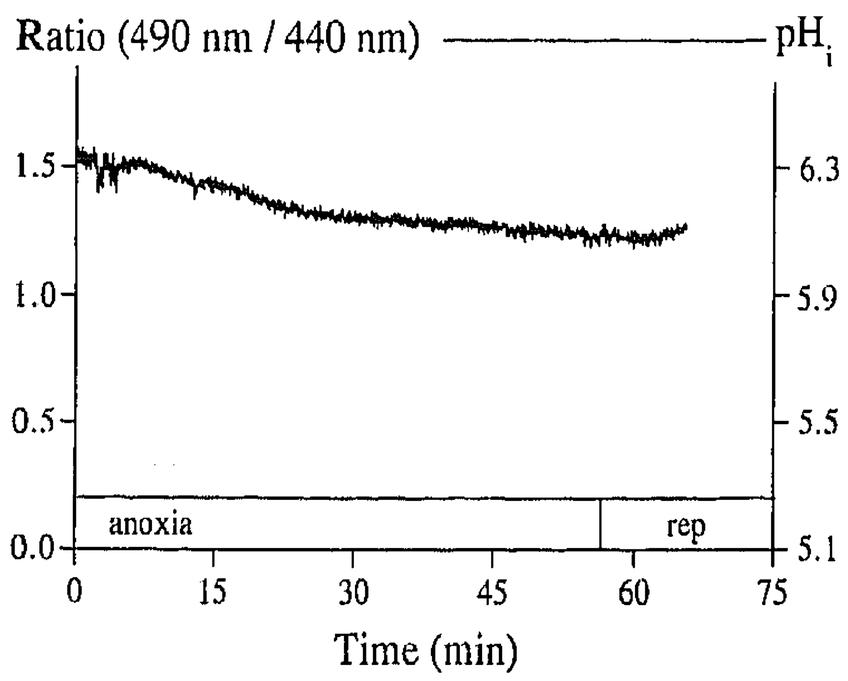

Fig. 4 Typical pH_i trace of a pH_i clamp of freshly isolated PT cells during anoxic incubation in substrate-free high-K^+ buffer of pH 6.0, containing 10 μM nigericin. During this pH clamp, the 490/440 nm ratio declines to 1.23 pH_i is presented as 490/440 nm ratios as well as real pH values

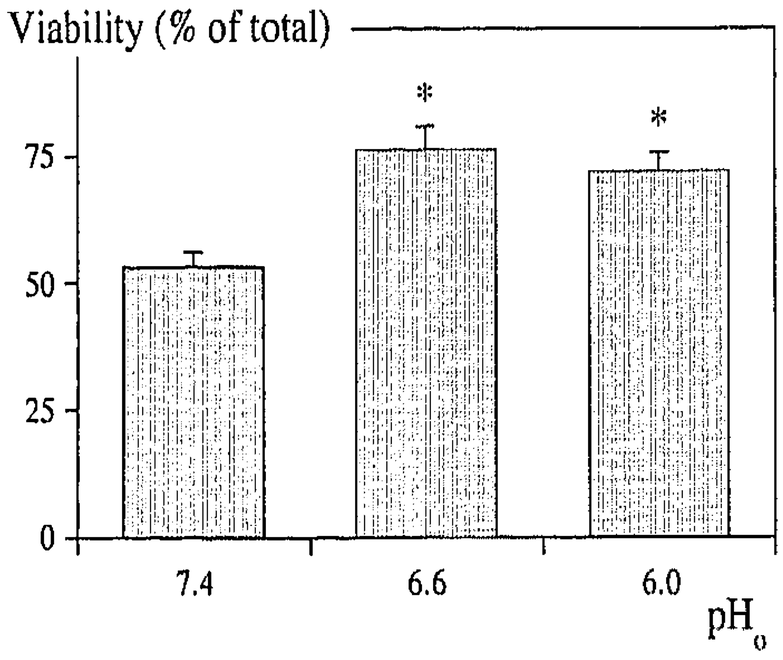

Fig. 5 Cell viability of freshly isolated PT cells after 60 min of anoxic incubation in substrate-free Krebs-Henseleit buffer (KHB) of pH 7.4 ($pH_o = 7.4$) or in substrate-free pH clamp buffer ($pH_o = 6.6$ and $pH_o = 6.0$). *Columns* represent mean viability values $\pm$ SEM with $N \geqslant 19$ (*$P < 0.05$ $pH_o = 6.6$ and 6.0 versus $pH_o = 7.4$)

6.0, in the presence of nigericin, cell viability was significantly higher than in the control situation (Fig. 5). Incubation of PT cells in a nigericin-free medium of pH 6.0 resulted in a pH_i of 6.8 $\pm$ 0.1 and a cell viability of 77% $\pm$ 4%, which is not significantly different from the results of experiments in which nigericin was present ($P > 0.1$). This control excludes an obscuring toxic effect of nigericin in the protection by cellular acidosis. In addition, we tried to clamp the pH_i of cultured PT cells during anoxia at 7.3 to see whether this protocol induced cell injury. However, pH_i measurements revealed that in cultured PT cells we were unable to clamp pH_i, since the cells still acidified during anoxia. Even in the presence of valinomycin or amiloride pH_i could not successfully be clamped at pH_i 7.3. Finally, cultured PT cells were incubated in a medium of pH 8.0 resulting in a pH_i of 7.3 $\pm$ 0.1. However, cell viability did not decrease during a 1-h anoxic incubation at a pH_o of 8.0. We did observe, however, a slight tendency for cells to

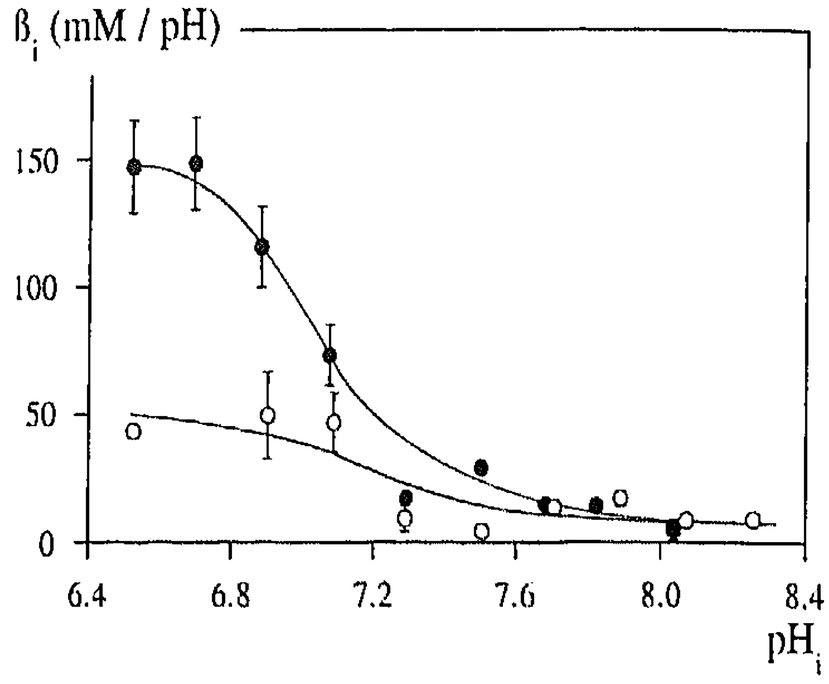

Fig. 6 Intrinsic buffering capacity (β_i) of cultured (○) and freshly isolated (●) PT cells. At $pH_i < 7.0$, β_i of freshly isolated PT cells is significantly higher than of cultured PT cells ($P < 0.05$). Results represent mean pH_i and β_i $\pm$ SEM with $N > 6$

start to detach themselves from the collagen coating, which could be an early sign of cell injury.

In Table 1 the influence of cytosolic acidosis on $[Ca^{2+}]_i$ is summarized for freshly isolated PT cells. It is shown that intracellular acidosis increased initial $[Ca^{2+}]_i$ significantly, but, on the other hand, maximal anoxic $[Ca^{2+}]_i$ was significantly reduced when compared to anoxia in the absence of acidosis. The mean $[Ca^{2+}]_i$ after 10 min of reperfusion was significantly higher than the initial level, independent of the pH_i during anoxia.

Estimation of β_i

A factor which could partly explain, in theory, the difference in pH_i values in cultured and freshly isolated PT cells during anoxic incubations is the β_i for both cell types; therefore, β_i was estimated. Figure 6 shows $ß_i$ values as a function of pH_i. In the pH range 8.0 to 7.3, $ß_i$ values were similar for cultured and freshly isolated PT cells. However, at lower pH_i, β_i values in freshly isolated PT cells started to increase and to deviate significantly from those measured in cultured cells. Nevertheless, the absence of cytosolic acidosis during anoxia in freshly isolated PT cells cannot be explained solely on the basis of a higher β_i at lower pH_i, but must be sought in the absence of glycolysis.

Discussion

The present study demonstrates that during anoxic incubation, intracellular acidosis develops in cultured PT cells, but does not occur in freshly isolated PT cells. Differences in cellular acidification must be due to differences in glycolytic capacities and cannot be explained by different β_i between cultured and freshly isolated PT cells. In addition, we have demonstrated

that intracellular acidosis is protective against anoxia-induced cell injury, since acidosis was protective in freshly isolated PT cells.

Studies using isolated perfused rat kidney have shown that acidosis, caused by ATP hydrolysis and accumulation of CO_2, protected against hypoxic cell injury [24]. Until now, in vitro experiments have demonstrated only a protective effect of extracellular acidosis against hypoxia/anoxia-induced cell injury in the kidney [7, 24, 26–29], hepatocytes [15] and cardiomyocytes [3]. In these studies, however, it was not shown that protection by low pH_o is mediated via intracellular acidosis. Our study demonstrates that anoxia caused intracellular acidosis in cultured, but not in freshly isolated, PT cells. Cultured cells, in general, are more glycolytic than are freshly isolated cells and, therefore, endogenous substrates at the onset of anoxia will be metabolized, forming lactic acid and ATP. In addition, hydrolysis of ATP leads to an intracellular acid load and, thus, to cytosolic acidification [8]. In contrast, non-cultured PT cells have less glycolytic capacity and do not form lactic acid during anoxia [9]. Finally, we showed that freshly isolated PT cells have a higher β_i at pH_i below 7.3 than do cultured PT cells, which could cause a slower development of acidosis, but cannot explain its absence. By subjecting freshly isolated PT cells to low pH clamp during anoxia, it could be shown that acidosis is also protective in these cells, obviously mediated by intracellular acidosis, since pH_i clamping experiments resulted in pH_i values of 6.7 and 6.1.

There are several mechanisms by which acidosis may protect against anoxia-induced cell death. First, hypoxia/anoxia-induced phospholipid degradation has been reported to be pH dependent [13]. Also ATP depletion, in chemical anoxia, resulted in a loss of phospholipid mass and increased permeability of the plasma membrane and cellular organelles [28]. Second, low pH has been shown to preserve metabolic energy in cardiomyocytes [14, 16]. In PTs, however, no increase in cellular ATP content was found during anoxia at a low pH_o, compared to a pH_o of 7.4 [15, 18, 26, 29]. In our experiments ATP preservation is also unlikely, since anoxia induced increases in $[Ca^{2+}]_i$ in cultured PT cells, in spite of cytosolic acidosis. Increases in $[Ca^{2+}]_i$ occur only after ATP depletion. Third, acidosis may preserve ionic gradients across the plasma membrane during anoxia. Cytosolic acidosis decreases plasma membrane conductance pathways and, as a result, cellular K^+ does not decrease during chemical anoxia in hepatocytes [6], or during anoxia in hepatocytes and rabbit PTs [19]. Another gradient which is influenced by pH is that of Ca^{2+} [26]. Under normal physiological conditions, pH_i and $[Ca^{2+}]_i$ appear to be closely linked; a decrease in pH_i reduces transmembrane Ca^{2+} fluxes in cardiomyoctyes and an increase in pH_i promotes Ca^{2+} -mediated processes during differentiation of the cell [26]. Low pH_i has also been shown to inhibit Ca^{2+} influx across the plasma membrane in kidney [24] and cardiomyocytes [14]. Burnier et al. [7] described elevated $^{45}Ca^{2+}$ uptake associated with cell injury during hypoxia in PTs. This hypoxia-induced cell injury could be prevented by acidosis which also abolished elevated $^{45}Ca^{2+}$ uptake. Others have reported that Ca^{2+} mediated damage caused by hypoxia can be inhibited by acidosis in a dose-dependent fashion, i.e. protection afforded by acidosis could be overcome by increasing perfusate Ca^{2+} concentration and the injury caused by elevated Ca^{2+} could be reduced by a further decrease in pH [23]. In the present study it was shown that acidosis slightly increased initial $[Ca^{2+}]_i$ levels in freshly isolated PT cells. This effect may be due to competition between H^+ and Ca^{2+} for intracellular Ca^{2+} binding sites. During anoxia, however, $[Ca^{2+}]_i$ levels were slightly reduced by exposure to acidosis. This result is in line with the notion that acidosis reduces Ca^{2+} influx across the plasma membrane, [7, 23], because we demonstrated previously that anoxia-induced increases in $[Ca^{2+}]_i$ resulted from Ca^{2+} influx [20, 21]. However, it is rather unlikely that the protective effect of acidosis is due to the small decrease in $[Ca^{2+}]_i$ levels. In freshly isolated PT cells it has been shown previously that anoxic cell injury and $[Ca^{2+}]_i$ levels are not directly related [21]. Ca^{2+} entry blockers such as D600 were shown to reduce anoxic $[Ca^{2+}]_i$ to normoxic levels with only small protective effects. In contrast, glycine protected against anoxic injury to the same extent as acidosis in the present study, but without an effect on anoxic $[Ca^{2+}]_i$ levels [21].

Finally, it has been shown that acidosis stabilizes cell membranes [1]. We have previously shown that 0.1 mM extracellular La^{3+} protects freshly isolated PT cells against anoxic injury via membrane stabilization [21]. The effect of acidosis in the present study and the effect of 0.1 mM La^{3+} in the previous study on cell viability are of similar magnitude.

In conclusion, cytosolic acidosis, which does develop in cultured, but not in freshly isolated, PT cells, cannot explain the differences in susceptibility to anoxic injury between cultured and freshly isolated PT cells.

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