Effects of Ca\(^{2+}\) channel blockers, low Ca\(^{2+}\) medium and glycine on cell Ca\(^{2+}\) and injury in anoxic rabbit proximal tubules

Ursula M. Rose, René J.M. Bindels, Jan W.C.M. Jansen, and Carel H. van Os

Department of Cell Physiology, University of Nijmegen, Nijmegen, and Department of Vascular Pharmacology, Solvay Duphar BV, Weesp, The Netherlands

Effects of Ca\(^{2+}\) channel blockers, low Ca\(^{2+}\) medium and glycine on cell Ca\(^{2+}\) and injury in anoxic rabbit proximal tubules. L-type Ca\(^{2+}\) channel blockers (CCBs) have been shown to be protective against ischemia-induced injury of the kidney, suggesting that increased intracellular Ca\(^{2+}\) levels (\([\text{Ca}^{2+}]_i\)) play an important role in the pathogenesis of ischemic cell injury. To assess the role of \([\text{Ca}^{2+}]_i\) in anoxic injury of the proximal tubule (PT) and the protective effect of CCBs, digital imaging fluorescence microscopy was used to monitor \([\text{Ca}^{2+}]_i\) in individual PT cells during 60 minutes of anoxia. \([\text{Ca}^{2+}]_i\) started to rise within 10 minutes and reached maximal levels between 30 to 45 minutes of anoxia. The onset of this increase and the maximal levels reached varied markedly among individual cells. The mean values for initial and maximal anoxic \([\text{Ca}^{2+}]_i\) were 109 ± 2 \(\mu\text{M}\) (\(N = 209\)) and 422 ± 14 \(\mu\text{M}\) (\(N = 240\)) \(\mu\text{M}\), respectively. Methoxyverapamil (D600; 1 \(\mu\text{M}\)) significantly reduced anoxic \([\text{Ca}^{2+}]_i\) to 122 ± 5 \(\mu\text{M}\) \(P < 0.05; N = 79\). Removal of extracellular Ca\(^{2+}\) completely abolished anoxia-induced increases in \([\text{Ca}^{2+}]_i\), confirming that these increases in \([\text{Ca}^{2+}]_i\), result from Ca\(^{2+}\) influx. During 60 minutes of anoxia, PT cells showed a gradual decrease in cell viability to 54 ± 2%. D600 (1 \(\mu\text{M}\)) significantly increased cell viability to 64 ± 3% \(P < 0.05\). Glycine (5 \(\mu\text{M}\)), however, increased cell viability to 77 ± 4% without a significant reduction in anoxic \([\text{Ca}^{2+}]_i\) levels. Low Ca\(^{2+}\) medium only protected when 0.1 mM La\(^{3+}\) was included, a condition which increased cell viability to 82 ± 5%. La\(^{3+}\) did not enter PT cells and probably protected via a membrane-stabilizing effect. The combination of glycine and La\(^{3+}\) did not further increase protection. In conclusion, D600 almost completely prevented anoxia-induced increases in \([\text{Ca}^{2+}]_i\), by blocking Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels. Since D600 only partly protected PT cells against anoxic injury, \([\text{Ca}^{2+}]_i\), unrelated cell injury, which is attenuated by glycine, is a more prominent factor in anoxia-induced cell injury in rabbit PT cells.

Disruption of intracellular Ca\(^{2+}\) homeostasis may be an important factor in the development of cell injury during ischemia, hypoxia or anoxia [1, 2]. Intracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)) has also been suggested to be important in hypoxic injury of renal proximal tubules (PTs) [3, 4]. Increases in \([\text{Ca}^{2+}]_i\), using ionomycin induced cell injury in cultured PT cells and in freshly isolated PTs [5, 6]. Others have argued that \([\text{Ca}^{2+}]_i\), is not a prerequisite for cell injury. Jacobs et al [7] observed cell injury in PTs during anoxia without significant changes in \([\text{Ca}^{2+}]_i\). In contrast, in cultured PT cells, chemical anoxia [8] as well as anoxia [9] increased \([\text{Ca}^{2+}]_i\), without detectable lactate dehydrogenase (LDH) release in the first 60 minutes of anoxia.

If \([\text{Ca}^{2+}]_i\), is a primary mediator of cell injury resulting from O\(_2\) deprivation, then prevention of Ca\(^{2+}\) entry into cells should prevent or delay cell death. Indeed, exposure of isolated PTs to 30 minutes of anoxia in a low Ca\(^{2+}\) medium reduced LDH release [10, 11], and delayed blebbing [8]. Moreover L-type Ca\(^{2+}\) channel blockers (CCBs) have been shown to reduce \([\text{Ca}^{2+}]_i\) uptake as well as LDH release after 10 minutes of hypoxic and anoxic incubation of PTs [12]. Although many studies have shown that CCBs can be protective against ischemic renal injury in various circumstances [4], there is no general agreement on the question whether CCBs directly protect renal epithelial cells [13]. So far, very high concentrations of CCBs have been used to show protective effects [11, 12]. These high concentrations preclude a specific effect on L-type Ca\(^{2+}\) channels and beneficiary effects may be related to nonspecific actions, as for example, membrane-stabilization [14]. In addition, L-type Ca\(^{2+}\) channels have not been demonstrated unequivocally in renal tubular cells [6]. A recent study on cell volume regulation of PT cells suggests the presence of L-type channels in these cells, since \([\text{Ca}^{2+}]_i\), was significantly reduced by 10 \(\mu\text{M}\) verapamil [15]. Most importantly, no direct study has been undertaken to investigate the effect of CCBs on \([\text{Ca}^{2+}]_i\) during hypoxia or anoxia in PT cells.

In a previous study, we developed a method to investigate increases in \([\text{Ca}^{2+}]_i\) in cultured PT cells induced by anoxic periods up to 60 minutes [9]. In that study complete absence of O\(_2\) was realized by inclusion of Oxyrase\(^{®}\), a mixture of oxygen species (ROS) to cellular injury which may occur in model studies using chemical anoxia or hypoxia [6]. In cultured PT cells, 1 \(\mu\text{M}\) methoxyverapamil (D600) significantly decreased anoxic \([\text{Ca}^{2+}]_i\), levels. However, the question whether reduction of anoxia-induced increase in \([\text{Ca}^{2+}]_i\), correlated with protection against cell injury could not be answered, since after 60 minutes of anoxia the cultured PT cells showed no significant increase in LDH release. It is known that in freshly-isolated PT cell injury occurs much faster [12]. Therefore, we studied anoxia-induced increases in \([\text{Ca}^{2+}]_i\), and the effects of CCBs in freshly-isolated PT cells to find out whether protective effects of CCBs on...
tubular cells are correlated with a reduction in anoxic [Ca²⁺]
levels.

**Methods**

**Isolation of proximal tubules**

Rabbit PT cells were isolated by immunodissection as described previously [9]. Briefly, kidneys were excised from New Zealand white rabbits (≈0.5 kg). A cortical cell suspension, obtained by enzymatic digestion of dissected cortical tissue, was incubated for 60 minutes on ice with monoclonal antibodies 85C8 and 101E12, recognizing PT cell surface specific antigens. After three washings, the cell suspension was added to goat anti-mouse IgG-coated petri dishes and incubated for 15 minutes at 20°C. The dishes were washed carefully and adherent PT cells were scraped off the dishes. The PT cells were collected and resuspended at a density of 1 × 10⁶ cells/ml in a mixture of Dulbecco’s Modified Eagle’s medium (Imperial #1-466-14, Hampshire, UK): Ham’s F12 medium (Gibco, #041-01765M, Paisley, UK) (1:1), supplemented with gentamycin (10 mg/ml), NaHCO₃ (25 mM), glutamine (14 mM), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (50 nM), O₂-, Na₂SeO₃ (50 nM) and 5% (vol/vol) fetal calf serum (FCS), pH 7.4 (K₁ + non-essential amino acids (Gibco, #043-01 140H, Paisley, UK), prostaglandin E₁ (70 ng/ml), triiodothyronine (5 pM), Na₂SeO₃ (50 nM) and 5% (vol/vol) fetal calf serum (FCS), pH 7.4 (K₁ + 5% FCS medium). PT cell suspension aliquots (5 ml) were incubated at 37°C in 25 cm² tissue culture flasks (Costar 3055, Cambridge, Massachusetts, USA) for maximally six hours in a gassed modified Krebs-Henseleit buffer [KHB; composition in mm: 138 NaCl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 1 l-alanine, 5 l-lactate, 20 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/tris-(hydroxy)methylaminomethane (HEPES/TRIS) and 360 mU/ml Oxyrase® (Oxyrase Inc., Ashland, Ohio, USA), pH 7.4]. It was mounted on the stage of a Nikon Diaphot inverted microscope (400× magnification) and [Ca²⁺]ᵢ, was measured using the MagiCal system. After 45 to 60 minutes of anoxic incubation, perforusion was started by perfusing the chamber with oxygenated KHB containing 10 mm glucose. For [Ca²⁺]ᵢ measurements during anoxia we used at least four preparations and from every preparation at least two coverslips were used. From one coverslip 8 to 16 cells could be monitored and analyzed, which was roughly the number of cells in the viewing field of the CCD camera (3 to 4 clumps of cells).

**Fura-2 loading of PT cells**

Clumps of freshly-isolated PT cells were attached to round coverslips (Ø22 mm; Menzel, Germany) which were coated with Cell-Tak® (Collaborative Research Incorporated, Bedford, Massachusetts, USA) as follows: 5 μl pure Cell-Tak® was brought onto the glass coverslip and dried in air for one hour at 20°C. Thereafter 100 μl of a PT cell suspension was added to the dried Cell-Tak® within 30 minutes at 37°C. The attached PT cells were loaded with fura-2 AM by incubating the coverslip with PT cells for one hour at 37°C in K₁ + 5% FCS medium containing 10 μM fura-2 acetoxymethyl ester (fura-2 AM; Molecular Probes, Eugene, Oregon, USA), 0.02% (wt/vol) pluronic F127 (Molecular Probes), 4% (vol/vol) FCS and 3 mm probenecid (Sigma Chemical Co., St. Louis, Missouri, USA). After loading, 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 leakage via organic anion transporters.

**Measuring [Ca²⁺]ᵢ**

[Ca²⁺]ᵢ in single cells was measured by a digital imaging technique making use of a MagiCal system (Applied Imaging Techniques, Tyne & Wear, UK). The fura-2-loaded PT cells were alternately excited at 340 and 380 nm and emitted light was captured at 510 nm with a CCD camera followed by digital imaging using TARDIS® software (Applied Imaging Techniques). Images at 340 and 380 nm were captured in one second followed by 30 seconds of no capturing, divided in 10 seconds excitation at 340 nm allowing for cell focusing, and 20 seconds of no excitation using a shutter to avoid bleaching. The cycle of one second capturing and 30 seconds of no capturing was repeated until the experiment was completed (~60 min). The MagiCal system has been described in detail by Neylon et al [16]. [Ca²⁺]ᵢ was calculated according to the formula derived by Grynkiewicz, Poenie and Tsien [17]: [Ca²⁺]ᵢ = Kᵣ X Rᵣ X [(R - Rᵣ)/(Rᵢ – Rᵣ)] where Kᵣ is the dissociation constant of fura-2 for Ca²⁺ of 224 nm; Rᵣ is the ratio of fluorescence of the cell at 340 and 380 nm; Rᵢ and Rᵣ represent the ratios of fura-2 fluorescence intensity at 340 and 380 nm excitation obtained by treating the PT cells with 5 μm ionomycin in the presence and absence [estimated by addition of 2 mm ethylene-glycolcolis(β-aminooethyl ether)-N,N,N’-tetaoacetic acid (EGTA)] of extracellular Ca²⁺, respectively, and Rᵣ is the maximal 380 nm signal divided by the minimal 380 nm signal.

**Anoxic chamber experiment**

To estimate [Ca²⁺]ᵢ during anoxia, the same anoxic chamber was used as described previously [9]. Briefly, fura-2-loaded PT cells attached to a coverslip were mounted in an anoxic chamber at 37°C. After filling the anoxic chamber with 100% N₂ gassed modified Krebs-Henseleit buffer [KHB; composition in mm: 138 NaCl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 1 l-alanine, 5 l-lactate, 20 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/tris-(hydroxy)methylaminomethane (HEPES/TRIS) and 360 mU/ml Oxyrase® (Oxyrase Inc., Ashland, Ohio, USA), pH 7.4]. It was mounted on the stage of a Nikon Diaphot inverted microscope (400× magnification) and [Ca²⁺]ᵢ, was measured using the MagiCal system. After 45 to 60 minutes of anoxic incubation, perforusion was started by perfusing the chamber with oxygenated KHB containing 10 mm glucose. For [Ca²⁺]ᵢ measurements during anoxia we used at least four preparations and from every preparation at least two coverslips were used. From one coverslip 8 to 16 cells could be monitored and analyzed, which was roughly the number of cells in the viewing field of the CCD camera (3 to 4 clumps of cells).

**Estimation of cell viability**

Clumps of PT cells attached to Cell-Tak®-coated coverslips were mounted in the anoxic chamber at 37°C in modified KHB. Simultaneously, partial O₂ pressure (PO₂) was monitored using a Clark-type oxygen electrode [9]. After 60 minutes of anoxic incubation, cell viability was estimated by means of Trypan blue or propidium iodide staining. To this end, the coverslip with PT cells was incubated for one to two minutes in 0.08% (wt/vol) Trypan blue or 5 μg/ml propidium iodide. The percentage stained cells was determined by means of light microscopy for Trypan blue staining or by means of fluorescence microscopy with excitation at 490 nm and 510 nm emission for propidium iodide staining. As controls, several coverslips with PT cells were incubated in the presence of oxygen and substrate. The influence of several conditions on cell viability was tested in such a way that for every condition at least four preparations were used. From every preparation at least two coverslips were incubated in the anoxic chamber, and after 60 minutes between 100 and 200 cells were counted after staining. The cells were chosen randomly by counting as many cells as possible on one coverslip within five minutes. We did not observe a specific pattern of cell killing within clumps of cells.
Figure 1. Time-dependency of increase in 
$[Ca^{2+}]_1$ in four individual PT cells (typical examples) in response to $O_2$ and glucose deprivation at 37°C. At −10 minutes the anoxic chamber is closed; 
$[Ca^{2+}]_1$ measurements start at 0 minutes (anoxia). After 32 minutes of anoxia, $O_2$ and glucose are reintroduced. In one cell a fall in the fluorescent ratio is observed after 30 minutes, due to loss of fura-2 fluorescence, indicative of cell death. The bold straight line represents a normoxic control experiment. $[Ca^{2+}]_1$ is presented as the 340 and 380 nm ratio of fura-2 excitation.

Materials

Conjugated antibodies were obtained from Sigma. D600 (methoxyverapamil) was kindly provided by Knoll AG (Ludwigshafen, Germany). Felodipine was donated by Astra Pharmaceutica BV (Rijswijk, NL). All chemicals were of the purest grade.

Statistical analysis

All reported data are expressed as means ± sd. Statistical analysis was performed on ratio values using analysis of variance ($P < 0.05$ is significant). Subsequently, statistical differences between experimental groups were estimated by means of contrast analysis according to Fisher [18].

Results

$[Ca^{2+}]_1$, during anoxia

As shown previously, $P_02$ in the anoxic chamber decreased rapidly as a result of filling the chamber with hypoxic medium [9]. The residual $P_02$ was reduced to zero within 10 minutes due to the enzyme complex Oxynase® [9]. As a result of substrate-free anoxia, $[Ca^{2+}]_1$ in freshly isolated PT cells started to rise and reached maximal levels within 45 minutes (Fig. 1). However, the onset of this rise and the maximal levels reached varied strikingly among individual cells within one preparation (Fig. 1) and subsequent preparations (Fig. 2). This heterogeneity was not only observed in cells from different clumps, but also within one clump. At the introduction of oxygen and glucose, elevated $[Ca^{2+}]_1$ declined rapidly towards initial levels, but in a few cells $[Ca^{2+}]_1$ remained elevated (Fig. 1). In some cells a sudden and abrupt decline in the fluorescence ratio was observed (Fig. 1). Inspection of the 340 and 380 nm fluorescence values indicated a decline in both signals, which means that leakage of fura-2 had occurred due to a loss of the cell membrane barrier.

In Figure 2, fura-2 ratios and $[Ca^{2+}]_1$ values are given which have been observed in close to 200 cells: the mean initial level, the individual maximal levels attained, and the mean value 10 minutes after reintroducing $O_2$ and substrate. $[Ca^{2+}]_1$ monitoring starts 10 minutes after filling the anoxic chamber due to mounting the chamber on the stage of the microscope. Since several cells lost fura-2 fluorescence during the experiment, the maximal stable fura-2 level, reached just before fura-2 started to leak, was used in calculating the mean anoxic fura-2 ratio. When the fura-2 level did not reach a plateau, then the ratio was excluded. From those cells which lost fura-2 during the anoxic incubation, the reperfusion fura ratios were also excluded. Therefore, Figures 2 through 7 also contain maximal anoxic fura-2 ratios of cells which did not survive the anoxic incubation. However, the fura-2 ratio was used which was reached just before the dye started to leak. We never observed a sudden increase in fura-2 ratio just before fura started to leak due to cell injury. The mean initial fura ratio ($1.01 ± 0.02$, $N = 218$) measured 10 minutes after closing the chamber, was not significantly different from the mean fura ratio ($0.98 ± 0.03$, $N = 209$, $P > 0.1$) 10 minutes after reperfusion.

Phenylalkylamines have been shown to reduce ischemic renal injury when present during the ischemic insult [19, 20], therefore we tested whether D600 reduces the rise in $[Ca^{2+}]_1$ during anoxia. Figure 3 shows that the presence of 1 μM D600 during the anoxic period reduced the maximal fura-2 ratio from $1.59 ± 0.06$ to $1.04 ± 0.04$ ($P < 0.05$). D600 also slightly, albeit significantly, reduced the initial fura-2 ratio from $1.01 ± 0.02$ to $0.89 ± 0.02$ ($P < 0.05$). A similar reduction in the ratio was observed 10 minutes after reperfusion: from $0.98 ± 0.03$ to $0.77 ± 0.03$. In addition, the effect of 1 μM felodipine was studied, but this dihydropyridine derivative was unable to significantly reduce the anoxia-induced increases in the fura-2 ratio (Fig. 4). When, however, the differences in fura-2 ratios between initial and the maximal anoxic levels were considered ($Δ$ ratio in...
Fig. 4. The effect of 1 μM felodipine on initial (basal), maximal anoxic, and reperfusion ratio values, and on Δ ratio values (maximal minus initial ratios). Experimental ratios (dashed columns) are compared to control ratios (open columns), that is, anoxic incubation without the addition of felodipine. Columns represent mean ratio values ± SE with N = 32 (*P < 0.05: felodipine vs. control ratio values).

Fig. 5. Effect of extracellular Ca²⁺ exclusion (−Ca⁺/EGTA) on initial (basal), maximal anoxic, and reperfusion ratio values, and on Δ ratio values (maximal minus initial ratios). Experimental ratios (dashed columns) are compared to control ratios (open columns), that is, anoxic incubation in the presence of 2 mM CaCl₂ without EGTA. Columns represent mean ratio values ± SE with N = 38 (*P < 0.05, experimental vs. control).

Next, the dependence of anoxia-induced increases in [Ca²⁺], on extracellular Ca²⁺ ([Ca²⁺]₀) was investigated. We studied two conditions, one in which [Ca²⁺]₀ was reduced below 10⁻⁸ M by adding 0.5 mM EGTA, and one in which Ca²⁺ was simply omitted from the solution. In the second condition, medium [Ca²⁺] was still ≈20 μM and therefore 0.1 mM La³⁺ was added to block any residual Ca²⁺ influx. Figures 5 and 6 clearly demonstrate that omitting [Ca²⁺]₀ or preventing Ca²⁺ influx by La³⁺ effectively reduced the anoxia-induced increase in [Ca²⁺], which proves that the rise in [Ca²⁺] stems from Ca²⁺ influx and not from depletion of intracellular Ca²⁺ stores.

Since glycine protects against ischemic injury, the effect of glycine on [Ca²⁺], during anoxia was also studied. The result is shown in Figure 7. Whatever the mechanism behind glycine protection may be, it is clear that glycine is unable to influence [Ca²⁺] during an anoxic period of 45 to 60 minutes.

Cell injury

In some cells loss of fura-2, indicative of cell death, was observed during the anoxic incubation. Therefore the effect of anoxia on cell viability was further quantified. Cell injury could not be assessed by measuring LDH-release because the number of cells in the anoxic chamber was too small to measure LDH-release reproducibly. For this reason, cell viability was
significantly increased cell viability up to 77.4% (P < 0.05). This significantly influence cell viability. Glycine during anoxia significantly increased cell viability by low Ca^2+ alone (data not shown). The effects of low Ca^2+ and of low Ca^2+ plus 0.1 mM La^3+ were also tested on the normoxic control group, but both conditions did not significantly influence cell viability. Glycine during anoxia significantly increased cell viability up to 77 ± 4% (P < 0.05). This protection occurs despite the elevated anoxic [Ca^{2+}]_i levels. The combination of glycine and 0.1 mM La^3+ further increased cell viability to 83 ± 7% (P < 0.05), albeit this further increase is not significant with respect to glycine or La^3+ alone (P > 0.1).

Figure 10 summarizes the observed effects of D600, Ca^{2+}-free solution and glycine on anoxia-induced increases in [Ca^{2+}]_i, and on cell viability after 60 minutes of anoxia. This figure demonstrates a clear dissociation between protective effects and effects on [Ca^{2+}]_i, suggesting a minor role for [Ca^{2+}]_i in the pathogenesis of anoxic injury of freshly-isolated PT cells.

### Discussion

In the present study, freshly-isolated PT cells were subjected to 60 minutes of anoxia. [Ca^{2+}]_i measurements showed anoxia-induced increases in [Ca^{2+}]_i, which were heterogeneous in onset and in the maximal level reached. The CCB, methoxyverapamil (1 μM) almost completely reduced the anoxia-induced increase in [Ca^{2+}]_i, but exhibited a moderate protective effect.

Cytosolic Ca^{2+} in PT cells could be measured conveniently after attachment of the cells to a coverslip with Cell-Tak®. This substance did not induce cell injury, but made it possible to follow single PT cells during an anoxic period of 60 minutes. Several individual PT cells within one preparation were monitored using digital imaging fluorescence microscopy. Some investigators have previously used cell suspensions [7, 12] in which responses of several thousands of tubules are summed. The present study demonstrated that anoxia-induced increases in [Ca^{2+}]_i, are rather heterogeneous at the single cell level. Nevertheless, maximal [Ca^{2+}]_i levels were always reached before 45 minutes of anoxia, with a mean value of 422 ± 14 nM (N = 240). In view of an extracellular Ca^{2+} concentration of 2 mM, this anoxic [Ca^{2+}]_i value represents a relatively modest increase. Anoxic incubation precludes mitochondrial Ca^{2+} loading due to the complete absence of oxidative phosphorylation and a driving force for Ca^{2+} uptake. Nonetheless, our results point to stabilization of the anoxic [Ca^{2+}]_i value below the extracellular level, suggesting the presence of some kind of endogenous autoprotective mechanism whereby the ATP-depleted state decreases the Ca^{2+} permeability of the plasma membrane, and prevents the cell from being flooded with Ca^{2+}. The anoxic [Ca^{2+}]_i value reported previously for cultured PT cells [9] was even higher than the one observed in freshly-isolated cells (662 ± 22 nM vs. 422 ± 14 nM). Other studies using cultured PT cells also reported modest increases in [Ca^{2+}]_i, after inhibition of metabolism [5, 21].

The complete suppression of anoxia-induced increases in [Ca^{2+}]_i, by removing [Ca^{2+}]_i, suggests that intracellular Ca^{2+} stores do not contribute to Ca^{2+} overload during anoxia. This phenomenon has also been observed in cultured PT cells [8, 9]. A possible explanation could be that when intracellular stores begin to release Ca^{2+}, the plasma membrane Ca^{2+}-ATPase may still operate at low ATP concentrations, and an early increase in [Ca^{2+}]_i, activates calmodulin and thereby stimulates the plasma membrane Ca^{2+}-ATPase.

The present study provides functional evidence for the presence of L-type Ca^{2+} channels in freshly-isolated PT cells. The phencylalkylamine D600 almost completely abolished anoxia-induced increases in [Ca^{2+}]_i, at a concentration of 1 μM. Previously, in cultured PT cells a reduction of [Ca^{2+}]_i, by 1 μM D600 has been shown [9], but the effect was less

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*Fig. 7. Effect of 5 mM glycine on initial (basal), maximal, and reperfusion ratio values and on ∆ ratio values (maximal minus initial ratios). The experimental ratios (dashed columns) are compared to control ratios (open columns), that is, anoxic incubation without addition of glycine. Columns represent mean ratio values ± SE with N = 77.*
profound, suggesting that L-type Ca\(^{2+}\) channels are either less important in anoxia-induced increases in [Ca\(^{2+}\)]\(_{i}\) or are less expressed in cultured PT cells. Felodipine, a dihydropyridine, was less effective than D600 which was also previously reported for cultured PT cells [9]. This finding confirms the observation of McCarty and O'Neil [15, 22], that verapamil but not nifedipine reduced resting [Ca\(^{2+}\)]\(_{i}\) in isolated PTs. Verapamil has been described to inhibit Na\(^+\)-Ca\(^{2+}\) exchange activity in cardiac sarcosomal membrane vesicles with an IC\(_{50}\) of approximately 200 \(\mu\)M [23]. Because in PT cells a complete reduction of anoxia [Ca\(^{2+}\)]\(_{i}\) is realized at 1 \(\mu\)M D600, inhibition of Ca\(^{2+}\) influx via Na\(^+\)-Ca\(^{2+}\) exchange is an unrealistic alternative. In addition, Na\(^+\)-Ca\(^{2+}\) exchange is most likely absent from PT cells, which was elegantly shown with molecular-biological techniques [24] and immunohistochemical techniques [25].

Almeida et al [12] recently reported that anoxia-induced increase in \(^{45}\)Ca uptake and LDH release in rat PTs was inhibited by verapamil during 10 minutes of anoxia. During longer anoxic incubations the time course of \(^{45}\)Ca uptake reached a plateau phase. We observed that D600 prevents increases in [Ca\(^{2+}\)]\(_{i}\), during 60 minutes of anoxia. Hence, the fura-2 imaging technique provides information over much longer experimental periods than conventional isotope uptake experiments.

Besides [Ca\(^{2+}\)]\(_{i}\) measurements, cell viability after 60 minutes of anoxia was determined. In contrast to cultured PT cells [9], freshly-isolated PT cells exhibit significant cell injury within 15 minutes of anoxia, which was demonstrated by Trypan blue or propidium iodide staining and loss of fura-2 fluorescence. This clearly demonstrates that freshly-isolated PT cells are more sensitive to anoxic injury than cultured PT cells. Differences in hypoxia tolerance have also been described between hepatoma cells and hepatocytes [26].

Exposure of PT cells to 60 minutes of anoxia in the absence of [Ca\(^{2+}\)]\(_{o}\) did not improve cell viability. Previously, Takano et al [10] reported that reduction of [Ca\(^{2+}\)]\(_{o}\), to around 2.5 \(\mu\)M...
attenuated cell injury in short-term anoxia of rabbit PTs. Either the longer anoxic incubation or the addition of EGTA may have resulted in loss of protection in our study. There is, however, no agreement whether lowering of [Ca\(^{2+}\)]\(_{i}\) offers protection [13]. For example, hepatocytes as well as cardiomyocytes were more susceptible to anoxic injury when [Ca\(^{2+}\)]\(_{i}\) was less than 10 \(\mu\)M [27, 28]. Surprisingly, addition of 0.1 mM La\(^{3+}\) to a nominally Ca\(^{2+}\)-free solution protected PT cells as effectively as glycine against anoxic injury. From the fura-2 ratio in Figure 6 it can be concluded that La\(^{3+}\) does not enter the cells during anoxic incubation, since fura-2 exhibits a similar response to La\(^{3+}\) as to Ca\(^{2+}\). Therefore, this novel protecting effect of small amounts of La\(^{3+}\) is exerted extracellularly, most likely via stabilization of the plasma membrane, resulting in a lower susceptibility to lysis by enzymes which are activated during anoxia.

Using PTs and cultured PT cells, Weinberg found that glycine protects against cell injury associated with chemical anoxia, ouabain and [Ca\(^{2+}\)]\(_{i}\) [6]. Also in our study, glycine provided protection in the absence of reducing anoxia-induced increases in [Ca\(^{2+}\)]\(_{i}\). Since the mechanism of this protection is still unknown, it is of interest that the protective effects of glycine and La\(^{3+}\) were not additive. This could mean that La\(^{3+}\) and glycine prevent a common factor from exerting its injurious effect. Hypothetically, when La\(^{3+}\) stabilizes the plasma membrane preventing enzymatic lysis then glycine could possibly inhibit those very enzymes which cause membrane lysis. Searching for such a common factor could be of help in uncovering the mechanism of glycine protection against cell injury.

In conclusion, anoxia-induced increases in [Ca\(^{2+}\)]\(_{i}\) in freshly-isolated PT cells results from Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels. Despite the fact that anoxia-induced increases in [Ca\(^{2+}\)]\(_{i}\) are almost completely suppressed by 1 \(\mu\)M D600, this CCB offers only moderate protection, suggesting a minor role of [Ca\(^{2+}\)]\(_{i}\) in the pathogenesis of anoxic cell injury.

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Reprint requests to C.H. van Os, Department of Cell Physiology, University of Nijmegen, Trigon Building, P.O.Box 9101, 6500 HB Nijmegen, The Netherlands.

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