Rise in Cytosolic Ca\textsuperscript{2+} and Collapse of Mitochondrial Potential in Anoxic, but Not Hypoxic, Rat Proximal Tubules\textsuperscript{1}

Susan M.A. Peters, Maria J.H. Tijsen, René J.M. Bindels, Carel H. Van Os, and Jack F.M. Wetzels\textsuperscript{2}

ABSTRACT
It has been suggested that ischemic renal proximal tubular cell injury is mediated by an increase in cytosolic calcium concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}). However, measurements of [Ca\textsuperscript{2+}]\textsubscript{i} in rat or rabbit proximal tubules exposed to hypoxia or anoxia have yielded ambiguous results. This study explored the possibility that the severity of oxygen deprivation and the energy state of the mitochondria are important determinants of [Ca\textsuperscript{2+}]\textsubscript{i}. To this end, [Ca\textsuperscript{2+}]\textsubscript{i} (measured with fura-2) and the mitochondrial membrane potential (measured with rhodamine 123) were studied simultaneously in individual rat proximal tubules in hypoxic and anoxic conditions. [Ca\textsuperscript{2+}]\textsubscript{i} did not change during hypoxia, but increased rapidly during anoxia. Increases in [Ca\textsuperscript{2+}]\textsubscript{i} were only observed in parallel with a decrease of rhodamine 123 fluorescence, which indicates a collapse of the mitochondrial membrane potential. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} during anoxia was prevented by incubating the tubules in a low Ca\textsuperscript{2+} medium, which did not interfere with the collapse of the mitochondrial membrane potential. Both hypoxic and anoxic incubation led to cell death, as assessed by the fluorescent dye propidium iodide. These results clearly demonstrate that the level of oxygen deprivation is critical in determining changes in [Ca\textsuperscript{2+}]\textsubscript{i}. Because cell damage occurred in both hypoxic and anoxic conditions, it was concluded that an increase in [Ca\textsuperscript{2+}]\textsubscript{i} is not a necessary prerequisite for the development of ischemic cell injury.

Key Words: Ischemia, hypoxia, anoxia, cytosolic calcium, mitochondrial membrane potential

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\textsuperscript{2} Correspondence to Dr. S.M.A. Peters, 162 Cell Physiology, PO Box 9100, 6500 HB Nijmegen, The Netherlands.

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The role of intracellular Ca\textsuperscript{2+} in ischemia-induced renal injury is still a matter of debate. Most evidence supporting a role for Ca\textsuperscript{2+} in ischemic renal injury is derived from experiments with Ca\textsuperscript{2+} channel blockers (1-4). It has been demonstrated that these agents afford protection against cell damage in various models of in vivo and in vitro ischemic renal injury. However, these studies can only provide indirect evidence for a role of Ca\textsuperscript{2+} in ischemic cell injury. Moreover, Ca\textsuperscript{2+} channel blockers could influence in vivo renal injury by their vasodilatory and diuretic effects (5). Furthermore, we recently demonstrated, in an in vitro model of ischemic tubular cell injury, that the protective effect of the Ca\textsuperscript{2+} channel blocker felodipine is independent of extracellular Ca\textsuperscript{2+} and is mimicked by felodipine analogs that do not block Ca\textsuperscript{2+} influx (6). These observations implicate that the role of Ca\textsuperscript{2+} in cell injury cannot be assessed by using Ca\textsuperscript{2+} channel blockers.

Evidence in favor of a role of increased cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) in ischemic injury stems from studies in which intracellular Ca\textsuperscript{2+} was measured. Using atomic absorption spectrophotometry, Takano and colleagues (7) observed increased levels of total cell Ca\textsuperscript{2+} during hypoxic incubation of rabbit proximal tubules. In rat proximal tubules, hypoxia caused an increase in the tubular uptake of Ca\textsuperscript{2+}, measured with the isotope \textsuperscript{46}Ca\textsuperscript{2+} (3). However, these studies have limitations, as they do not discriminate between influx of Ca\textsuperscript{2+} into the cytosol and binding of Ca\textsuperscript{2+} to cell membranes. Also, these experiments do not give information about the intracellular free Ca\textsuperscript{2+} concentration. Finally, no evidence is provided that the abnormalities in Ca\textsuperscript{2+} handling precede rather than result from cell injury. With the advent of newer technologies that allow measurements of [Ca\textsuperscript{2+}]\textsubscript{i}, with the use of fluorescent probes, it has become possible to more precisely characterize changes in [Ca\textsuperscript{2+}]\textsubscript{i} in relation to hypoxic cell injury. However, the results of several studies have been ambiguous. Kribben \textit{et al.} (8) demonstrated a rapid increase in [Ca\textsuperscript{2+}]\textsubscript{i}, during hypoxic incubation of rat proximal tubules, which preceded and correlated to ensuing cell injury. Rose \textit{et al.} (9) observed heterogeneous responses of [Ca\textsuperscript{2+}]\textsubscript{i} to anoxia in isolated rabbit proximal tubular cells and could not demonstrate a correlation between [Ca\textsuperscript{2+}]\textsubscript{i} and cell injury. Weinberg \textit{et al.} (10) found that rabbit proximal tubules subjected to chemical anoxia did not develop increases of [Ca\textsuperscript{2+}]\textsubscript{i} until just before loss of viability. Finally, Jacobs \textit{et al.} (11) showed that [Ca\textsuperscript{2+}]\textsubscript{i} did not increase in rabbit proximal tubules during anoxia or chemical hypoxia.

So far, the discrepancies between these studies have remained unexplained. However, it has been
suggested that the exact level of oxygen deprivation might be important. Takano et al. (7) observed that total cell Ca$^{2+}$ increased during hypoxia but decreased slightly during complete anoxia. Based on these observations, it was hypothesized that the differences in Ca$^{2+}$ levels can be fully explained by differences in the accumulation of Ca$^{2+}$ by the mitochondria (7,12,13). Thus, the severity of hypoxia, which determines the capacity of mitochondria to accumulate Ca$^{2+}$, could influence total cell Ca$^{2+}$ and [Ca$^{2+}$]. This hypothesis has not been tested so far. Therefore, we designed experiments to measure [Ca$^{2+}$] and the mitochondrial membrane potential simultaneously in individual rat proximal tubules under hypoxic or anoxic conditions, with fura-2 and rhodamine 123 (14), respectively. [Ca$^{2+}$] did not change during hypoxia, but increased rapidly during anoxia. [Ca$^{2+}$], only increased when the mitochondrial membrane potential collapsed. However, hypoxia as well as anoxic incubation led to cell death.

**MATERIALS AND METHODS**

**Isolation of Proximal Tubules**

Proximal tubules were isolated from male Sprague-Dawley rats weighing 200 to 250 g as previously described in detail (3,4). Briefly, rats were anesthetized with 0.1 mL/100 g body wt of pentobarbital (Narcoret, Aphaora, Arnhem, The Netherlands), and the kidneys were flushed with 40 mL of an adapted Krebs-Henseleit buffer (Buffer A) containing 0.17 mg/mL Collagenase A (Boehringer-Mannheim, Mannheim, Germany) and 0.42 mg/mL hyaluronidase (Boehringer-Mannheim). Buffer A was kept on ice (4°C) and gassed with 95% O$_2$/5% CO$_2$, respectively. [Ca$^{2+}$], did not change during hypoxia, but increased rapidly during anoxia. [Ca$^{2+}$], only increased when the mitochondrial membrane potential collapsed. However, hypoxia as well as anoxic incubation led to cell death.

**Experimental Setup**

Two different Leiden chambers were used, called Chambers A and B. Chamber A was used for superfusion experiments and closely resembles the ones used by Kribben et al. (16). It allowed for controlled superfusion and contained specific in- and outlets for gassing, fluid, and thermometer. However, in this chamber, complete anoxia cannot be achieved. To accomplish anoxic levels, we had to perform no-flow experiments and use Oxyrase (Oxyrase Inc., Ashland, OH). For these experiments, a standard Leiden chamber (Chamber B) was used, supplemented with an insert like that in Chamber A to optimize the exchange of in- and outcoming fluid and to limit perturbation (16). Superfusion could take place in the so-called open mode, and the chamber could be closed air-tight thereafter. The chambers were heated by different mechanisms. Temperature in Chamber A was controlled by heating the incoming perfusate by means of a water coat. Chamber B was placed in an adaptable metal heating block. Both methods created a temperature of 35°C to 36°C in the chamber.

Superfusion experiments were performed in Chamber A. Gassed Buffer A (95% O$_2$/5% CO$_2$ for normoxic buffer, 95% N$_2$/5% CO$_2$ for hypoxic buffer) was pumped through Viton tubing (aperture 1 x 3 mm; Rubber BV, Hilversum, The Netherlands) into the chamber. An HPLC gas-tight switch was used to switch between buffers. The buffer in the chamber was gassed over with the corresponding gas mixture (16). Loaded tubules were mounted on the stage of the microscope and flushed for 5 min with normoxic buffer. After an additional period of superfusion of 5 min with normoxic buffer (normoxic experiments) or hypoxic buffer (hypoxic experiments) to equilibrate the chamber, measurements were started.

The experiments with Oxyrase (Oxyrase Inc., Ashland, OH) and their controls were performed in Chamber B with Buffer A. After the final period of loading, Chamber B was inserted in the heating block on the stage of the microscope. Tubules were flushed for 5 min with normoxic buffer and equilibrated for 5 min with normoxic buffer (normoxic experiments) or with...
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The chamber was filled with buffer and closed. When appropriate, oxynase or EGTA was added just before the chamber was closed. Measurements were then started.

One should realize that in a closed chamber leakage of fura-2 could influence the observed [Ca\(^{2+}\)] signal. To circumvent these problems, we used additional experimental approaches. In one, we added glycine (2 mM) to the final incubation buffer to prevent cell injury and dye leakage as much as possible. Also, we used chamber B filled with a thin layer of fluid, which was continuously gassed over. In this experimental setting, gassing could be switched from N\(_2\) to O\(_2\) without changing the fluid content.

**Measurements with Fluorescent Probes**

Fura-2-loaded tubules were excited alternately with 340 nm and 380 nm. The emitted light was filtered through a 490-nm filter and captured by a CCD camera. Reduced nicotinamide adenine dinucleotide (NADH) fluorescence was measured at 380 nm excitation and 490 nm emission with unloaded tubules. Rhodamine 123 was measured with 490 nm excitation combined with a 510-nm-emission filter. When fura-2 and rhodamine 123 were measured simultaneously, all fluorescence was captured at 510 nm. Because of the intense rhodamine 123 fluorescence, neutral density (ND) filters were switched during the simultaneous fura-2 and rhodamine 123 measurements. With a ND-1.5 filter for fura-2 and a ND-2 filter for rhodamine 123, similar fluorescence intensities were captured. Propidium iodide (PI) was used as a marker for cell death. This membrane-impermeant dye can only stain the nucleus once the plasma membrane is irreversibly damaged. For superfusion experiments, PI (Sigma, 5 \(\mu\)g/mL) was added to a separate bottle of Buffer A. This PI-containing buffer was perfused for 90 s, followed by a 60-s flushing period with the original buffer to get rid of any residual PI. Fluorescence was then captured. To obtain the 100% reference signal, we perfused with 70% ethanol for 60 s, with PI-containing buffer for 90 s, and with PI-free buffer for 60 s. Cell death was expressed as the percentage of the maximal (100%) fluorescence signal, with TARDIS software (Joyce Loeble, Tyne & Wear, UK). For PI measurements during no-flow experiments, PI was injected close to the adhered tubules and was allowed to stain the nuclei for 90 s. Then tubules were flushed and images were captured. These computer images of PI-stained tubules were saved, and processed later with NIH-Image (public domain), and stained nuclei were counted afterward. PI was measured with 490 nm excitation and 510 nm emission wavelengths. All emitted light was captured by a CCD camera followed by digital imaging with TARDIS software (Joyce Loeble, Tyne & Wear, UK). Calibrations of fura-2 ratios were performed with ionomycine and EGTA. However, tubules often were severely injured by these substances, which seriously hampered the calibration, as described previously by others (17,18). When calibrations were successful, we obtained a \(R_{\text{max}}\) of approximately 3.6 (10 \(\mu\)M ionomycine) and a \(R_{\text{min}}\) of approximately 0.4 (5 mM EGTA). With these values, basal [Ca\(^{2+}\)] was calculated to vary between 60 and 150 nM. However, throughout this publication, we use fura-2 ratios to represent [Ca\(^{2+}\)].

**Chemicals**

All chemicals were of the purest grade and obtained from Sigma or Merck (Darmstadt, Germany) unless otherwise indicated.

**RESULTS**

**NADH Fluorescence**

NADH fluorescence measured during normoxic superfusion remained constant. When tubules were superfused with hypoxic buffer, NADH levels rose rapidly (Figure 1). On reoxygenation, NADH levels returned to basal values (Figure 1). The experimental setup of the no-flow experiments precluded NADH measurements during the onset of anoxia or hypoxia. However, on reoxygenation, a decrease of the NADH signal occurred, thus suggesting that NADH levels had been increased.

**[Ca\(^{2+}\)] Measurements**

During normoxic superfusion, [Ca\(^{2+}\)], presented as the fura-2 ratio, remained constant (Table 1). When hypoxic buffer was used, also no increase in [Ca\(^{2+}\)] was observed (Table 1 and Figure 2). The [Ca\(^{2+}\)] dependence of the fura-2 ratio signal was confirmed by the addition of the Ca\(^{2+}\)-ionophore ionomycine and the Ca\(^{2+}\)-chelating agent EGTA as shown in Figure 2. The addition of ionomycine (10 \(\mu\)M) always resulted in a rapid increase in the fura-2 ratio, which was reversed by the subsequent addition of EGTA (5 mM).

The no-flow experiments yielded similar results. During both normoxia and hypoxia, no increase in

![Figure 1](image-url)  
Figure 1. NADH increase during hypoxic superfusion. Un-loaded tubules were superfused with gassed buffer (95% N\(_2\)/5% CO\(_2\)) in an adapted Leiden chamber (see Methods). NADH fluorescence was measured at 380 nm excitation and 490 nm emission with digital imaging fluorescence microscopy. Hypoxia increased NADH levels, which was quickly reversible on reoxygenation. Figure represents typical trace of a single rat proximal tubule.

Statistics

All reported data are expressed as means ± SEM. Statistical analysis was performed with analysis of variance, and comparisons between groups were done by the Student-Newman Keuls test with INSTAT software (Graphpad Corp., San Diego, CA), unless stated differently. A P value <0.05 was considered significant.
TABLE 1. Levels of cytosolic calcium in rat proximal tubules incubated under normoxic or hypoxic conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fura-2 Ratio</th>
<th>0 min</th>
<th>10 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>0.97 ± 0.03</td>
<td>0.98 ± 0.03</td>
<td>0.99 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1.00 ± 0.02</td>
<td>0.99 ± 0.02</td>
<td>0.97 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

a Cytosolic free calcium levels were measured in individual fura-2-loaded rat proximal tubules using digital imaging fluorescence microscopy (see Methods). Tubules were superfused with either normoxic (95% O2/5% CO2) or hypoxic (95% N2/5% CO2) buffer in a customized Leiden chamber. Fura-2 ratios (340 nm/380 nm) are given as means ± SEM. Means are derived from measurements of 32 individual tubules from two preparations (hypoxia) and 24 tubules from three preparations (normoxia).

Figure 2. (Ca2+)i during hypoxic superfusion. Fura-2-loaded tubules were perfused with hypoxic buffer (95% N2/5% CO2) in an adapted Leiden chamber with digital imaging fluorescence microscopy (see Methods). (Ca2+)i, as represented by the fura-2 ratio (340 nm/380 nm), remained unchanged during hypoxic superfusion. Appropriate increases and decreases of (Ca2+)i could be observed after the addition of 10 μM ionomycin and 5 mM EGTA, respectively. Figure represents typical trace of a single rat proximal tubule.

Figure 3. (Ca2+)i during anoxia. Fura-2-loaded tubules were studied in a no-flow Leiden chamber (see Methods) with digital imaging fluorescence microscopy. Anoxia was induced by the addition of 1.2% Oxyrase. (Ca2+)i was detected (Table 2). In contrast, the addition of Oxyrase, which results in complete anoxia, caused a rapid increase in the fura-2 ratio (Table 2 and Figure 3). On reoxygenation, (Ca2+)i, rapidly decreased (fura-2 ratio 1.57 ± 0.06 and 1.08 ± 0.08 for 20-min anoxia versus reoxygenation, respectively; 69 and 34 tubules from at least five preparations; P < 0.001; see Figure 3). When tubules were made anoxic in the presence of 2 mM glycine, the fura-2 ratio after 10 min of anoxia was 1.48 ± 0.04 and returned to 1.15 ± 0.03 on reoxygenation (P < 0.0001, paired t test; see Figure 4, A and B).

In separate experiments, tubules were incubated in the closed chamber, which was filled with a thin layer of fluid (see Methods). In this setting, reoxygenation could be achieved by changing from N2/CO2 to O2/CO2 without changing the bathing fluid. The results of these experiments also demonstrated a reversible increase in (Ca2+)i (fura-2 ratio 1.49 ± 0.03 versus 1.23 ± 0.02 for anoxia versus reoxygenation, P < 0.0001, paired t test, see Figure 5, A and B).

Mitochondrial Membrane Potential

During normoxic and hypoxic superfusion, rhodamine 123 fluorescence remained virtually constant (0.65 ± 0.13 and 0.61 ± 0.12% loss of initial fluorescence/min for normoxia and hypoxia, respectively; 14 and 22 tubules of at least two preparations, respectively; not significant [NS]). As shown in Figure 6A, both the fura-2 ratio and rhodamine 123 fluorescence were unaffected by 20 min of hypoxia. The no-flow

TABLE 2. Levels of cytosolic calcium in rat proximal tubules incubated under normoxic, hypoxic, or anoxic no-flow conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fura-2 Ratio</th>
<th>0 min</th>
<th>10 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>1.07 ± 0.03</td>
<td>1.12 ± 0.02</td>
<td>1.11 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Hypoxia</td>
<td>1.10 ± 0.03</td>
<td>1.11 ± 0.02</td>
<td>1.03 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Anoxia</td>
<td>1.13 ± 0.02</td>
<td>1.49 ± 0.04b,c,d</td>
<td>1.57 ± 0.06b,c,d</td>
<td></td>
</tr>
</tbody>
</table>

a Cytosolic free calcium levels were measured in individual fura-2-loaded rat proximal tubules using digital imaging fluorescence microscopy (see Methods). Tubules were studied in a no-flow Leiden chamber. Anoxia was induced by the addition of 1.2% Oxyrase. Fura-2 ratios are given as means ± SEM. Means are derived from measurements of at least 49 tubules from five preparations (0 min and 10 min) or 21 tubules from three preparations (20 min).
b P < 0.001 versus normoxia.
c P < 0.001 versus hypoxia.
d P < 0.001 versus hypoxia.
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Figure 4. (A) ([Ca\textsuperscript{2+}]\textsubscript{i}) during anoxia with glycine. Fura-2–loaded tubules were studied in the presence of 2 mM glycine in a no-flow Leiden chamber (see Methods) with digital imaging fluorescence microscopy. Anoxia was induced by the addition of 1.2% Oxyrase. ([Ca\textsuperscript{2+}]\textsubscript{i}), as represented by the fura-2 ratio, rapidly increased during anoxia, which was reversible on reoxygenation. The addition of glycine had no effect on ([Ca\textsuperscript{2+}]\textsubscript{i}). Figure represents typical trace of a single rat proximal tubule. (B) Separate fura-2 traces during anoxia with glycine. Fura-2–loaded tubules were studied in the presence of 2 mM glycine in a no-flow Leiden chamber (see Methods) with digital imaging fluorescence microscopy. Anoxia was induced by the addition of 1.2% Oxyrase and induced a rapid increase in the 340-nm signal (bold line) and a concurrent decrease in the 380-nm signal (dotted line), with a reversal on reoxygenation. These traces also demonstrate that there is no apparent loss of total fura-2 signal. Figure represents typical traces of a single rat proximal tubule.

Figure 5. (A) ([Ca\textsuperscript{2+}]\textsubscript{i}) during "thin-layer" anoxia. Fura-2–loaded tubules were incubated in a no-flow Leiden chamber (see Methods), which was filled with a thin layer of fluid, and studied with the use of digital imaging fluorescence microscopy. Anoxia was induced by the addition of 1.2% Oxyrase. ([Ca\textsuperscript{2+}]\textsubscript{i}), as represented by the fura-2 ratio, rapidly increased during anoxia. In this setting, reoxygenation could be performed simply by switching the gassing. ([Ca\textsuperscript{2+}]\textsubscript{i}) rapidly decreased on reoxygenation. Figure represents typical trace of a single rat proximal tubule. (B) Separate fura-2 traces during "thin-layer" anoxia. Fura-2–loaded tubules were incubated in a no-flow Leiden chamber (see Methods), which was filled with a thin layer of fluid, and studied with the use of digital imaging fluorescence microscopy. Anoxia was induced by the addition of 1.2% Oxyrase and provoked a rapid increase in the 340-nm signal (bold line) and a concurrent decrease in the 380-nm signal (dotted line). In this setting, reoxygenation could be performed simply by switching the gassing. This resulted in a rapid reversal of both signals. There is no apparent loss of total fura-2 signal. Figure represents typical traces of a single rat proximal tubule.

Experiments gave similar results: during both normoxic and hypoxic incubation of tubules in Chamber B, rhodamine 123 fluorescence remained constant (0.01 ± 0.12 and 0.73 ± 0.09% loss of initial fluorescence/min for normoxia and hypoxia, respectively; 19 and 21 tubules of at least two preparations, respectively; NS). In contrast, the addition of Oxyrase led to a prompt decrease of rhodamine 123 fluorescence levels (7.77 ± 0.52% loss of initial fluorescence/min; 46 tubules from eight preparations; P < 0.001 versus normoxic and hypoxic no-flow). The fall in rhodamine...
Figure 6. (A) \( [\text{Ca}^{2+}]_i \) and mitochondrial membrane potential during hypoxic superfusion. Tubules were loaded simultaneously with fura-2 AM and rhodamine 123 and studied with the use of digital imaging fluorescence microscopy (see Methods). The bold line represents the fura-2 ratio and the dotted line the rhodamine 123 fluorescence, depicting \( [\text{Ca}^{2+}]_i \) and mitochondrial membrane potential, respectively. Both signals remained stable during the period of hypoxic superfusion. Figure represents typical trace of a single rat proximal tubule.

(B) \( [\text{Ca}^{2+}]_i \) and mitochondrial membrane potential during anoxia. Tubules were loaded simultaneously with fura-2 AM and rhodamine 123 and studied in a no-flow Leiden chamber with the use of digital imaging fluorescence microscopy (see Methods). Anoxia was induced by the addition of 1.2% Oxyrase. The bold line represents the fura-2 ratio and the dotted line the rhodamine 123 fluorescence, depicting \( [\text{Ca}^{2+}]_i \) and mitochondrial membrane potential, respectively. Anoxia induced a collapse of the mitochondrial membrane potential and a concomitant increase in \( [\text{Ca}^{2+}]_i \). Figure represents typical trace of a single rat proximal tubule.

123 fluorescence always coincided with an increase in \( [\text{Ca}^{2+}]_i \) levels (Figure 6B). To check the interdependence of the \( [\text{Ca}^{2+}]_i \) signal and the mitochondrial membrane potential, we manipulated both parameters separately. The addition of ionomycin (10 \( \mu \)M) to normoxic tubules led to a rapid increase in \( [\text{Ca}^{2+}]_i \), but did not result in a fall in rhodamine 123 fluorescence (Figure 7). The anoxia-induced \( [\text{Ca}^{2+}]_i \) increase could be blocked completely by the addition of EGTA (5 mM), but rhodamine 123 fluorescence still declined rapidly (Figure 8). The subsequent addition of 10 mM \( \text{CaCl}_2 \) evoked a prompt increase in \( [\text{Ca}^{2+}]_i \) (Figure 8).
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Viability

Cell death, as assessed by the percentage of PI staining, amounted to 25 ± 2% (29 tubules from two preparations) after 20 min of normoxic superfusion. Superfusion with hypoxic buffer increased mortality to 63 ± 4% after 20 min (31 tubules from three preparations; P < 0.001 versus normoxic superfusion, unpaired t test). Unexpectedly, we observed major cell injury even in normoxic tubules incubated in the closed chamber. Moreover, no differences existed between the number of PI-stained nuclei of normoxic, hypoxic or anoxic tubules after 20 min of incubation (59 ± 5%, 47 ± 4%, and 45 ± 4% for normoxia, hypoxia, and anoxia, respectively; at least 38 tubules from four preparations; NS). Hence, viability of tubules incubated under normoxic no-flow conditions was lower than during normoxic superfusion experiments. We performed control no-flow experiments in which the normoxic buffer in the chamber was replaced every 5 min with fresh, oxygenated, warm buffer. Viability, as assessed by the percentage of PI-stained nuclei, then amounted to 31 ± 4% (27 tubules from three preparations, P < 0.001 versus normoxic no-flow, P < 0.05 versus hypoxic and anoxic no-flow), and was comparable to the values obtained with normoxic superfusion. The addition of 2 mM glycine to Buffer A protected anoxic no-flow tubules from lethal cell damage (14 ± 2% PI-stained nuclei after 10 min; 27 tubules from two preparations).

DISCUSSION

Although the role of Ca2+ in oxygen-deprivation injury has been debated for many years, no consensus has yet been reached as to whether cytosolic Ca2+ levels ([Ca2+]i) actually increase during hypoxia. So far, increases in [Ca2+]i (8,9,17) as well as stable levels (11) have been reported. Takano et al. (7) demonstrated that differences in the level of hypoxia influenced total cell Ca2+. Based on these data, a hypothesis was put forward (7,12,13), suggesting that changes in total cell Ca2+ and [Ca2+]i could be dependent on the mitochondrial energy state.

Based on this concept we have studied the changes in [Ca2+]i under hypoxic and anoxic conditions, and related these changes to alterations in the mitochondrial membrane potential. To this end, freshly isolated rat proximal tubules were loaded with fura-2 AM, rhodamine 123 and PI, to measure [Ca2+]m, mitochondrial membrane potential and cell death, respectively. NADH fluorescence was measured in unloaded tubules as a quick and reliable measure of the impairment of oxidative phosphorylation, i.e., of hypoxia (8).

With the use of a superfusion system with gas-tight tubing, we were able to achieve hypoxia as revealed by an increase in NADH fluorescence. Although this degree of hypoxia was severe enough to cause cell death, [Ca2+]i was not affected. The superfusion setup did not allow measurements at complete anoxic conditions. Pilot studies performed with the use of a very sensitive O2-electrode (Gesellschaft für Medizinische Sondentechnik, Riel, Germany) showed that the O2 tension during hypoxic superfusion in the chamber was between 5.5 and 6.5 mm Hg. To achieve anoxia, we used a closed chamber and no-flow conditions. Anoxia was established by using the commonly accepted enzyme system Oxyrase (3,17), which consumes oxygen and causes complete anoxia within minutes. In this closed chamber, no increase in [Ca2+]i occurred when Oxyrase was omitted, a situation similar to that with the hypoxic superfusion. However, after addition of Oxyrase, we observed rapid increases in [Ca2+]i. This elevation in [Ca2+]i preceded and was not caused by cell death, because reoxygenation of the anoxic tubules resulted in a rapid return to basal [Ca2+]i levels. To exclude possible artifacts caused by leakage of fura-2 under no-flow conditions, we performed two sets of control experiments. First, the established cytoprotective agent glycine (19,20) was used under anoxic no-flow conditions. Glycine (2 mM) prevented cell death but had no effect on the increase in [Ca2+]i. Second, mere reoxygenation of anoxic tubules without reperfusion also resulted in a return to basal [Ca2+]i, thus showing that washout of leaked fura-2 is not responsible for the observed decrease in [Ca2+]i. Altogether, these data clearly demonstrate a difference in [Ca2+]i handling between hypoxic and anoxic tubules.

Subsequently, we studied the relation between changes in [Ca2+]i, and the mitochondrial membrane potential. To this end, proximal tubules were loaded simultaneously with fura-2 AM and rhodamine 123. Under normoxic and hypoxic conditions, only a very slow loss of rhodamine 123 fluorescence was observed. However, when Oxyrase was added, rhodamine 123 fluorescence rapidly decreased, representing a collapse of the mitochondrial membrane potential. Such a decrease in rhodamine 123 fluorescence always coincided with an increase in [Ca2+]i.

Thus, our data not only demonstrate differences in Ca2+ handling between hypoxic and anoxic tubules, but also show a correlation between [Ca2+]i and the energetic state of the mitochondria. We propose that during hypoxia mitochondria remain energized and thus capable of accumulating Ca2+, whereas anoxia results in complete deenergization of the mitochondria and hence loss of their ability to retain Ca2+. As a consequence, anoxia, in contrast to hypoxia, causes an increase in [Ca2+]i, which is in line with previous speculations (7,12,13).

High levels of [Ca2+]i are reported to be deleterious for mitochondrial functioning (21). Consequently, it should be considered that a high level of [Ca2+]i itself might provoke a collapse of the mitochondrial membrane potential. Therefore, we have performed several control experiments. The addition of ionomycin to normoxic tubules resulted in a rapid increase in [Ca2+]i. In contrast, rhodamine 123 fluorescence levels showed the same slow decrease which is characteristic for normoxic and hypoxic tubules. Thus, an
increase in \([\text{Ca}^{2+}]_i\) per se does not influence the mitochondrial membrane potential. Also, we added the Ca\(^{2+}\)-chelating agent EGTA together with Oxyrase. No increase in \([\text{Ca}^{2+}]_i\) was observed, but rhodamine 123 fluorescence still decreased remarkably quickly, again indicating that the collapse of the mitochondrial membrane potential occurs independently of a rise in \([\text{Ca}^{2+}]_i\). The experiments performed with the use of EGTA confirm previous evidence (8) that increases in \([\text{Ca}^{2+}]_i\) during anoxia result from Ca\(^{2+}\) influx from the extracellular compartment.

Several observations made in this study may help to explain the conflicting data reported in the literature. First, it has become clear that complete anoxia is very difficult to achieve in a superfusion system. Even though gas-tight tubing was used and the fluid in the chamber was continuously gassed over with 95% N\(_2\)/5% CO\(_2\), O\(_2\) tension in Chamber A could not be brought below 5.5 mm Hg. The only group of researchers who observed \([\text{Ca}^{2+}]_i\) increases during hypoxic superfusion of rat proximal tubules are situated in Denver (8), where the atmospheric pressure is considerably lower. Jacobs et al. (11), who measured rabbit proximal tubules in perfused cuvettes, reported stable \([\text{Ca}^{2+}]_i\), which is in line with our hypoxic experiments. These authors referred to their experiments as “anoxic” and defined anoxia by an increase in NADH fluorescence. However, the present study shows that NADH accumulation is not a useful indicator of anoxia, because NADH fluorescence also increased during hypoxia. Altogether, it is very likely that the apparently conflicting data reported in the literature relate to differences in severity of oxygen deprivation. From our data, we cannot derive an exact relationship between increases in \([\text{Ca}^{2+}]_i\) and the prevailing PO\(_2\) value. Future experiments should be directed at measuring PO\(_2\) more precisely in superfusion systems.

In view of the cell injury that occurred in the closed chamber during normoxia, we cannot draw conclusions on the role of \([\text{Ca}^{2+}]_i\) in inducing lethal anoxic cell injury. A detailed discussion of this aspect is, however, beyond the scope of our paper. We may conclude that \([\text{Ca}^{2+}]_i\) does not play a role in hypoxic injury, because hypoxia resulted in increased PI staining, despite low and stable levels of \([\text{Ca}^{2+}]_i\). Therefore, other mechanisms must be responsible, as has been suggested before (6,8–10). The addition of glycine during anoxia protected tubules despite increases in \([\text{Ca}^{2+}]_i\). This confirms studies demonstrating that glycine protects renal tubules independently from changes in \([\text{Ca}^{2+}]_i\) (22,23). Further studies conducted with improved superfusion techniques that enable anoxic superfusion will be required to unravel the role of Ca\(^{2+}\) in anoxic renal injury.

In conclusion, we present the first simultaneous observations of \([\text{Ca}^{2+}]_i\) and mitochondrial membrane potential in individual rat proximal tubules during hypoxia and anoxia. Superfusion experiments showed that the achieved level of hypoxia was severe enough to increase NADH levels rapidly and cause cell death, whereas both \([\text{Ca}^{2+}]_i\) and the mitochondrial membrane potential remained stable. Anoxia induced a simultaneous loss of the mitochondrial membrane potential and an increase in \([\text{Ca}^{2+}]_i\). Thus, this study not only demonstrates differences in Ca\(^{2+}\) handling between hypoxic and anoxic tubules, but also shows a correlation between these differences and the energetic state of the mitochondria. We propose that during hypoxia energized mitochondria are capable of accumulating Ca\(^{2+}\), whereas completely deenergized anoxic mitochondria lose their ability to retain Ca\(^{2+}\), which results in a rapid increase in \([\text{Ca}^{2+}]_i\).

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