Na-P_i cotransporter type I activity causes a transient intracellular alkalinization during ATP depletion in rabbit medullary thick ascending limb cells

F. Jans, M. Ameloot, P. Wouters, and P. Steels

Abstract: The cellular pathophysiology of renal ischemia–reperfusion injury was investigated in primary cell cultures from rabbit medullary thick ascending limb (MTAL). Metabolic inhibition (MI) was achieved with cyanide and 2-deoxyglucose. Sixty minutes of MI caused a profound but reversible decrease in intracellular concentration of ATP ([ATP]_i). Intracellular pH (pH_i) first decreased after initiation of MI, followed by a transient alkalinization. When [ATP]_i reached its lowest value (<1% of control), the cells slowly acidified to reach a stable pH_i of 6.92 after 50 min of MI. In the presence of EIPA (10 μmol/L), the pattern of changes in pH_i was unchanged and acidification was not increased, indicating that the Na⁺/H⁺ exchangers were inactive during ATP depletion. When inorganic phosphate (P_i) or Na⁺ was omitted from the apical solutions during MI, the transient alkalinization was no longer observed and the cytosol slowly acidified. Experiments on Na⁺-dependent alkalinizations revealed the presence of a Na–P_i cotransporter in the apical cell membrane. With indirect immunofluorescence, the Na–P_i cotransporter expressed in these primary cell cultures could be identified as Na–P_i type I. Although the exact physiological role of Na–P_i type I still is unresolved, these experiments demonstrate that apical Na–P_i type I activity is increased at the onset of ATP depletion in MTAL cells.

Key words: MTAL, cell culture, ischemia, metabolic inhibition, intracellular pH, alkalinization, Na–P_i cotransporter.

Résumé : On a examiné la pathophysiologie cellulaire de la lésion d'ischémie-reperfusion rénale dans des cultures de cellules primaires provenant de la branche ascendante large médullaire (BALM). On a induit une inhibition métabolique (IM) au moyen de cyanure et de 2-désoxyglucose. Après 60 min, l'IM a causé une inhibition profonde mais réversible de la [ATP]_i. Après le déclenchement de l'IM, il y a eu une diminution du pH_i, suivie d'une alcalinisation transitoire. Lorsque que la [ATP]_i a atteint sa valeur minimale (<1 % de la valeur témoin), les cellules se sont acidifiées lentement pour atteindre un pH_i stable de 6,92 après 50 min. En présence d'EIPA (10 μmol/L), le profil des modifications du pH_i est demeuré inchangé et l'acidification n'a pas augmenté, ce qui indique que les échangeurs Na⁺/H⁺ ont été inactifs durant la déplétion de l'ATP. En l'absence de P_i ou de Na⁺ dans les solutions apicales durant l'IM, l'alcalinisation transitoire a disparu et le cytosol s'est acidifié lentement. Les expériences sur les alcalinisations dépendantes du Na⁺ ont révélé la présence d'un cotransporteur Na–P_i dans la membrane cellulaire apicale. L'immunofluorescence indirecte a permis d'identifier que le cotransporteur Na–P_i exprimé dans ces cultures de cellules primaires est le Na–P_i de type I. Le rôle physiologique exact du Na–P_i de type I n'est toujours pas connu; toutefois, ces expériences démontrent que l'activité apicale de ce cotransporteur augmente au début de la déplétion de l'ATP dans les cellules de la BALM.

Mots-clés : culture cellulaire, ischémie, inhibition métabolique, pH intracellulaire, alcalinisation, cotransporteur Na–P_i.

[Traduit par la Rédaction]

Introduction

Injury to tubular cells plays a central role in ischemic acute renal failure (ARF). Although the vast majority of studies on the pathophysiology of ischemic tubular cell injury have focused on proximal tubular cells, several lines of

evidence suggest an important role for cells of the medullary thick ascending limb (MTAL) in the pathophysiology of ARF (Brady et al. 2000; De Greef et al. 2003; Gobé et al. 1999; Ysebaert et al. 2000). It is therefore surprising to find only one publication in which cellular ischemia of MTAL cells has been explored. The authors reported a slow increase of intracellular Ca²⁺ concentration ([Ca²⁺]_i) towards an apparent plateau phase (approximately 470 nmol/L) with a rapid decline to preischemic levels upon reperfusion (Rose et al. 1994). To our current knowledge, no other data on the cellular pathophysiology of ischemia in MTAL cells are available. Furthermore, no measurements of intracellular pH (pH_i) during ischemia in MTAL cells or distal tubular cells in general can be found. Therefore the goal of this study is to investigate the time course of changes in pH_i during ischemia in MTAL cells. In this study the ischemic conditions

Received 21 February 2007. Published on the NRC Research Press Web site at cjpp.nrc.ca on 20 December 2007.

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were simulated by pharmacological ATP depletion, indicated further on as metabolic inhibition.

To investigate ischemic injury of MTAL cells a new method for obtaining very pure primary cell cultures from MTAL segments has been developed. These primary cell cultures were shown to retain both morphological and functional characteristics of their *in vivo* counterparts (Jans et al. 2000).

Materials and methods

Solutions and chemicals

For optimal control of extracellular pH, HEPES-buffered (HCO_3^- -free) balanced salt solutions were used (HB-BSS). HB-BSS contained (in mmol/L): 128 NaCl, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 L-alanine, 5 glycine, 10 glucose, 20 HEPES. The solution for MI contained (in mmol/L): 2.5 Na^+ -cyanide (NaCN), 10 2-deoxyglucose (2-DOG), 128 NaCl, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 L-alanine, 5 glycine, 20 HEPES. For reperfusion the standard HB-BSS was used. Na^+ -free solutions contained (in mmol/L): 100 *N*-methyl-D-glucamine (NMDG^+), 100 HCl, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 KH_2PO_4 , 1 L-alanine, 5 glycine, 10 glucose, 20 HEPES. Na^+ -free solutions for metabolic inhibition contained KCN instead of NaCN. Final pH of all these solutions was brought to 7.4 with (solid) Tris and osmolality to 320 mosm/kg H_2O with mannitol. All chemicals were from Sigma-Aldrich Chemical Company (St. Louis, Mo.).

Cell culture

Methods and materials for obtaining primary cell cultures from rabbit MTAL segments and for cell culture of TALH-SVE cells have been previously published (Jans et al. 2000). The TALH-SVE cells were immortalized cells derived from rabbit MTAL cells that were kindly supplied by Professor R.K.H. Kinne (Max Planck Institute für Molekulare Physiologie, Dortmund, Germany).

All measurements were performed on cells grown in cell culture inserts with 0.33 cm^2 collagen-coated polytetrafluoroethylene membranes, pore size 0.4 μm (Transwell-COL, Costar, Corning Inc., N.Y.).

ATP measurements

Confluent monolayers of cells grown on permeable filter supports (Transwell-COL, Costar, Corning Inc.) were washed and equilibrated in HB-BSS solution for 1 h. At the start of the recovery phase, cell layers were washed 3 times with HB-BSS containing glucose or pyruvate or both. Control cells were incubated for the same amount of time in BSS. ATP measurements were performed with a luciferin-luciferase-based assay kit (Molecular Probes, Invitrogen, Eugene, Ore.). The reaction buffer contained 150 $\mu\text{g} \cdot \text{mL}^{-1}$ luciferin, 1.25 $\mu\text{g} \cdot \text{mL}^{-1}$ luciferase, 5 mmol/L MgSO_4 , 1 mmol/L dithiothreitol (DTT), 25 mmol/L Tricine buffer, 0.1 mmol/L EDTA, and 0.1 mmol/L azide, at pH 7.8. The cells were solubilized in 650 μL of somatic cell ATP-releasing agent (Sigma-Aldrich, St. Louis, Mo.) for 30 s. Fifty microlitres of cell extract was added to 450 μL of reaction buffer. ATP levels were measured with a luminometer type 1250 from Wallac (Turku, Finland). At the start of

each new series of ATP measurements, a calibration curve was obtained with standard ATP solutions (Molecular Probes, Invitrogen, Eugene, Ore.) in the concentration range 10^{-8} – 10^{-4} mol/L. The results are presented as a percentage change compared with control, a primary cell culture that was kept in HB-BSS for the same time as the ischemia–reperfusion time of the sample.

Microfluorescence setup

The fluorescence measurements were performed with a home-built microfluorimeter under computer control. Cell culture inserts with confluent monolayers were placed in a home-built Ussing-type chamber that was mounted on the stage of a Nikon TMD 35 inverted microscope (Tokyo, Japan) and were continuously superfused on both sides with HB-BSS at 37 °C. The perfusion speed was adjusted in such a way that the apical and basolateral volume of the bath was completely exchanged in 1 min. Electronically controlled valves (080T4 Mixing Valve, Bio-Chem Valve Inc., Boonton, N.J.) were used to change between gravity-fed perfusing solutions. An end-mounted vacuum line, placed just above the fluid level, removed excess perfusate. The Ussing-type chamber, the perfusion solutions, perfusion tubing, and mixing valves were all located inside a home-built transparent box that was constructed around the stage of the inverted microscope. The air inside the box was kept at 37 °C by constant air flow in a closed circuit, containing a heating element under control of a configurable loop controller (Gefran 1000, Provaglio d'Iseo, Italy) connected to a thermocouple inside the box.

A 40×/0.60 objective (LD Achroplane, Carl Zeiss, Oberkochen, Germany) was used. The light of a 100 W Xenon lamp (Nikon, Tokyo, Japan) was filtered for excitation by interference filters and was reflected towards the cells by a dichroic mirror. The filters were placed in a computer-controlled filter wheel, Lambda-10 (Sutter Instrument Company, Novato, Calif.). Neutral density filters, inserted between the microscope and the filter wheel, reduced the intensity of the source. For pH_i measurements, the fluorescence emission, after passing through an interference filter, was detected with a charge-coupled device (CCD) camera. The Quantix CCD camera (Photometrics, Tucson, Ariz.) was equipped with a Kodak KAF 1400 CCD (grade 2, MPP) with 1317×1035 pixels and cooled to -25 °C by a thermoelectric cooler. The exposure time was 5 s. The acquisition of pairs of images was performed every minute and was controlled by a homemade program based on the V for Windows software (Digital Optics, Auckland, New Zealand). The signals were obtained by spatially integrating the pixels over the cells. The background image was automatically subtracted, pixel by pixel, from the image of the loaded cells.

Measurement of intracellular pH

Confluent primary cell cultures were placed in a home-built Ussing-type chamber. After the background signals were measured (Schneckenburger et al. 1996), the cells were loaded from the apical and the basolateral side by exposure to the membrane-permeant form of the fluorescent pH indicator BCECF (Molecular Probes, Invitrogen, Eugene, Ore.) at a concentration of 10 $\mu\text{mol/L}$ using a stock solution

of 5 mmol/L in DMSO. Loading was performed for 15 min at 37 °C and for an additional 75 min at room temperature in HB-BSS containing 1% BSA. After loading, excessive dye in the apical and basolateral solution was washed out for at least 10 min.

The excitation light was filtered at 440 nm and 490 nm by the interference filters 440DF20 and 490DF20 from Omega Optical (Brattleboro, Vt.) and was reflected towards the cells by the dichroic mirror 515DRLPXR from Omega Optical. The fluorescence emission was monitored at 535 nm using the interference filter 535DF25 from Omega Optical.

Calibration of the BCECF fluorescence ratio F_{490}/F_{440} versus a given pH_i was performed at the end of each experiment, using the nigericin/high- K^+ technique (Thomas et al. 1979). Cells were clamped at 3 different pH levels (6.5, 7.0, and 7.5) by using calibration solutions at 37 °C containing 13 μ mol/L nigericin and 140 mmol/L K^+ . This K^+ concentration closely approximates the reported cytosolic K^+ concentration of rabbit TALH cells (Eveloff et al. 1980). For the considered pH range, a linear calibration function was used.

Indirect immunofluorescence for Na- P_i cotransporter type I and type II

Polyclonal antibodies raised in rabbits against a C-terminal peptide of sodium and inorganic phosphate (Na- P_i) type I and type II cotransporters were obtained from the laboratory of H. Mürer (Institute of Physiology, University of Zurich, Switzerland). These antibodies were shown to cross-react with the rabbit isoform of type I (Na- P_i 1 protein) for immunohistochemistry and for Western blotting (Biber et al. 1993; Verri et al. 1995) and with the rabbit isoform of type II (Na- P_i 6 protein) for Western blotting (Custer et al. 1994; Verri et al. 1995), respectively. Confluent primary cell cultures were washed with PBS and fixed with methanol (100%) for 15 min at room temperature. After washing with PBS, the cells were permeabilized and blocked by incubation for 20 min at room temperature with PBS containing Triton X-100 (0.2%) and normal goat serum (NGS, 10%). After washing, the cells were incubated for 1 h at room temperature with the primary antibody at various dilutions in PBS containing 1% NGS and 0.025% sodium azide. After washing 3 times with PBS, cells were incubated for 1 h at room temperature with the secondary antibody (goat anti-rabbit IgG) conjugated with Alexa Fluor 488 (20 μ g/mL) (Molecular Probes, Invitrogen, Eugene, Ore.) in PBS containing 1% NGS and 0.025% sodium azide. After washing and mounting in glycerol (100%), the cell culture inserts were placed on coverslips and examined on the stage of the microfluorescence microscope using a narrow-band filter system for Alexa Fluor 488 (excitation 495 nm, emission >515 nm) from Omega Optical.

Statistical analysis

Data from ATP and pH_i measurements were reported as means $\pm$ SE. Initial acidification-alkalinization rates were obtained from fitting linear equations to the recorded pH_i time courses. Data from pH_i measurements were converted to proton concentrations to calculate the mean $\pm$ SE and for statistical analysis. For uniformity, all graphical data were presented as pH_i and not $[H^+]_i$.

Fig. 1. Relative changes in ATP concentration during 1 h of metabolic inhibition followed by 1 h of reperfusion. Values at different time points are expressed relative to the ATP concentration of time-matched controls. Each point represents the mean relative ATP concentration of 3 different primary cell cultures. ($\blacklozenge$), reperfusion with pyruvate (10 mmol/L), $n = 3$; ($\blacksquare$), reperfusion with glucose (10 mmol/L), $n = 3$.

Statistical significance was judged from a one- or two-sided Wilcoxon test. Paired and unpaired Student's t test were used only after checking for normality assumptions, as indicated in the text. Significance was accepted at $p < 0.05$. All tests are performed with the statistical package StatXact (version 3.0.1) (Cytel Software, Cambridge, Mass.).

Results

ATP measurements

All data are expressed relative to the ATP content of control primary cell cultures. The controls were time-matched, so the relative ATP content after 60 min of MI and 60 min of recovery was compared with the ATP content of cells that were incubated for 2 h in HB-BSS. Every single ATP measurement represents the ATP content of one confluent primary cell culture. As shown in Fig. 1, the ATP content declined very rapidly after initiation of MI. After 60 s only $46\% \pm 5\%$ ($n = 3$) of the initial ATP content was left. After 10 min of MI only $1.7\% \pm 0.1\%$ ($n = 3$) of initial ATP content remained. A further drop to $0.4\% \pm 0.1\%$ ($n = 3$) was observed after 15 min. ATP levels remained below 1% of the original value during the rest of the period of MI.

pH_i measurements

In a first series of experiments, pH_i was measured by using the same protocol of metabolic inhibition as for ATP measurements (Fig. 2). During the first 4 min of ATP depletion, the cytosol started to acidify. From 4 to 10 min after initiation of MI, however, the cytosol transiently alkalinized. This alkalinization was followed by a slow acidification to reach the lowest pH_i value of 6.9 ± 0.1 ($n = 4$) after 50 min of ATP depletion, after which it stabilized. Upon reperfusion, pH_i remained at this acidic value for 4 min, followed by a small and transient further acidification, after which pH_i started a slow recovery to reach preischemic values after 20 min.

Fig. 2. Changes in pH_i during 60 min of metabolic inhibition with NaCN and deoxyglucose (DOG). Reperfusion was performed by washing out NaCN and DOG and by the re-addition of glucose to the apical and basolateral perfusion solutions. Values are means $\pm$ SE, $n = 4$.

Fig. 3. Changes in pH_i in TALH-SVE cells during 60 min of metabolic inhibition with NaCN and deoxyglucose (DOG). Reperfusion was performed by washing out NaCN and DOG and by the re-addition of glucose to the apical and basolateral perfusion solutions. Values are means $\pm$ SE, $n = 3$.

Because the transient alkalinization during the first phase of metabolic inhibition was unexpected, immortalized TALH-SVE cells were subjected to the same protocol to exclude artifacts inherent to the experimental setup. As shown in Fig. 3, changes in pH_i in TALH-SVE cells consisted of a slow acidification during metabolic inhibition followed by a slow and complete pH_i recovery upon reperfusion. Therefore, the results obtained with the primary cell cultures were related to the specific phenotype of these cells.

Since increased activity of Na^+/H^+ exchangers (NHE) might be responsible for the initial alkalinization, a series of MI experiments was performed in the presence of 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (10 $\mu\text{mol/L}$). This concentration of EIPA effectively blocks all NHE activity in these primary cell cultures (data not shown). The results from 5 different experiments are summarized in Fig. 4.

Also in the presence of EIPA, a transient alkalinization during the first 20 min of MI was observed. In its absolute value, this alkalinization (expressed as $\Delta[\text{H}^+]_i$) was even larger, although p was not significant. The subsequent acidification also was not different from the acidification in the absence of EIPA ($p = 0.73$), although it had not reached a stable value at the end of the ischemia period. EIPA was not added to the reperfusion solutions.

In a next series of experiments, the effects of CN^- and DOG on pH_i were investigated separately. Inhibition of the mitochondrial respiratory chain by CN^- initially caused the cells to acidify, rapidly followed by a transient alkalinization, after which the cytosol slowly acidified from pH_i 7.23 ± 0.04 to pH_i 7.19 ± 0.03 ($n = 4$). Although by the end of the 60-minute ischemia period (at which point ATP content was 81% of the starting value, data not shown), pH_i had

Fig. 4. Changes in pH_i during 60 min of metabolic inhibition with NaCN (2.5 mmol/L) and deoxyglucose (DOG) (10 mmol/L). 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (10 μ mol/L) was added to the apical and basolateral ischemia solution. Values are means $\pm$ SE, $n = 4$.

Fig. 5. Changes in pH_i during inhibition of the mitochondrial respiratory chain with NaCN (2.5 mmol/L). Values are means $\pm$ SE, $n = 4$.

only reached its preischemic value, the cells further alkalinized upon wash-out of CN^- to $pH_i 7.26 \pm 0.03$ ($n = 4$) (Fig. 5). In Fig. 6, the data of 4 experiments in which glycolysis was blocked with DOG are summarized. Under these circumstances, at the end of the 60-minute ischemia period, 19% of the intracellular ATP content was left (data not shown). Immediately after the addition of DOG, a transient alkalinization of the cytosol took place, followed by an acidification to $pH_i 6.84 \pm 0.02$ ($n = 4$) at 60 min, without reaching a stable value. The total change in $[H^+]_i$ during the alkaline transient was not different from the alkaline transient during CN^- -induced energy depletion.

In a next series of experiments, the effect of P_i during MI was explored. If P_i was omitted from the apical perfusion solution, a transient alkalinization was no longer observed: the change in pH_i during ATP depletion consisted of a simple acidification after initiation of MI and a slow recovery upon reperfusion (Fig. 7). Since this indicated that HPO_4^{2-}

influx through the apical cell membrane was responsible for the alkaline transient that was observed in all previous experiments, the effect of P_i on pH_i in these primary cell cultures was investigated under nonischemic conditions.

Sudden removal of P_i from either the apical or the basolateral perfusion solution did not affect pH_i . Sudden addition of P_i to the basolateral perfusion solution did not change pH_i . In contrast, the addition of 2 mmol/L P_i to the apical perfusion solution alkalinized the cells from $pH_i 6.84 \pm 0.09$ to $pH_i 7.18 \pm 0.08$ ($n = 10$) (Fig. 8). The initial alkalinization rate was 0.09 ± 0.03 pH units/min. Addition of phosphate in the absence of Na^+ (reverse activity of the apical Na^+/H^+ exchanger was prevented by the addition of EIPA 10 μ mol/L to all apical solutions) did not result in an intracellular alkalinization ($n = 5$) (Fig. 8).

Finally, a series of MI experiments was performed in the absence of Na^+ in the apical solutions. Reverse activity of the apical Na^+/H^+ exchanger again was prevented by the ad-

Fig. 6. Changes in pH_i during inhibition of anaerobic glycolytic ATP production with deoxyglucose (DOG) (10 mmol/L) in the absence of glucose. Values are means $\pm$ SE, $n = 4$.

Fig. 7. Changes in pH_i during 60 min of metabolic inhibition with NaCN (2.5 mmol/L) and deoxyglucose (DOG) (10 mmol/L). P_i was omitted from the apical ischemia solution. Values are means $\pm$ SE, $n = 4$.

dition of EIPA (10 $\mu\text{mol/L}$) to all apical solutions. Resting pH_i in these conditions was 6.95 ± 0.06 ($n = 4$). At the start of MI (KCN was used to replace NaCN), pH_i immediately started to fall and stabilized after 40 min at pH_i 6.45 ± 0.03 ($n = 4$). This acidification was significantly greater than the acidification observed with all previous protocols ($p < 0.05$). Again, a complete return of pH_i towards resting value was observed upon reperfusion despite the absence of Na^+ in the apical reperfusion solution (Fig. 9).

Immunofluorescence

Since Na-P_i cotransport activity was restricted to the apical cell membrane, indirect immunofluorescence for both “epithelial” subtypes of Na-P_i cotransporters, that is, Na-P_i 1 (rabbit type I) and Na-P_i 6 (rabbit type II), was performed: a fluorescent cellular staining pattern was obtained only with anti-type I antibodies (dilution 1/200). Control ex-

periments in which the primary antibody was omitted, were negative (Fig. 10).

Discussion

Changes in ATP

After the start of MI, the intracellular ATP content in these primary cell cultures decreased very rapidly (it dropped to less than 50% after only 60 s and to less than 2% after 10 min of MI). For comparison, in MDCK cells (an immortalized cell line with several properties of distal tubular cells) ATP content decreased to 30% after 5 min of MI with NaCN and DOG (McCoy et al. 1988). In freshly isolated distal (cortical) tubular cells that were subjected to MI with iodoacetate and KCN, ATP concentration decreased to 20% after 30 min (Lash et al. 1996). The most likely explanation for this difference is the presence of a very

Fig. 8. At time 7 min (arrow), phosphate (2 mmol/L) was added to the apical superfusion solution (■) (means + SE, $n = 10$). At time 7 min (arrow), phosphate (2 mmol/L) was added to the sodium-free apical superfusion solution (◆) (means - SE, $n = 5$).

high energy turnover in the primary cultured cells. Na^+ , K^+ ATPase activity has been reported to be quite high in CTAL and MTAL, so that the turnover rate of the ATP pool is very fast (ATP half-life is about 4–5 s) (Morel and Doucet 1992). To exclude that the rapid decrease of the ATP content in these primary cultured MTAL cells was an artifact, the effect of NaCN and DOG on the luciferin-luciferase reaction was tested and no interference was observed.

Transient intracellular alkalinization during metabolic inhibition

In most cells exposed to ischemia and hypoxia, pH_i decreases (Burke and Schrier 1997). Although no pH_i measurements in MTAL cells have been published, experiments on MDCK and A6 cells in our laboratory indicated that also in these distal tubular cells, a gradual acidosis develops during MI (S. Despa and I. Smets, unpublished observations). Therefore, the finding of a transient alkalinization during the initial phase of ATP depletion was unexpected. However, some reports on (transient) alkalinizations during ATP depletion have been published previously. During ischemia in muscle and myocardial cells, phosphocreatine (PCr) breakdown usually results in a transient alkalinization at the onset of ischemia (Allen et al. 1985; Eisner et al. 1989; Fry et al. 1987; Kemp et al. 2001). PCr is a high-energy phosphate donor and plays an important role not only in muscle contraction but also in buffering intracellular energy storage. Rat MTAL cells possess a large amount of PCr (approaching that of the brain) (Takeda et al. 1994). However, in contrast to myocardial cells where ATP content starts to decline only after PCr concentration has dropped dramatically (Fry et al. 1987), in rat MTAL cells PCr and ATP concentration decrease in parallel (Bastin et al. 1987). Since PCr breakdown will only alkalinize the cells as long as ATP levels remain elevated and since ATP concentration declines very rapidly in these primary cultures (even before the onset of the alkalinization) it can be concluded that the alkalinization observed is not the result of PCr breakdown.

In 1999, Jorgensen and collaborators (Jorgensen et al. 1999) observed alkalinizations following an initial acidifica-

tion during chemical anoxia (azide in the presence of glucose) in primary cell cultures from mouse neocortical neurons. In the presence of EIPA only a simple acidification was observed. Therefore, the authors concluded that activation of the NHE was responsible for the alkalinization. Since in the present experiments a transient alkalinization was observed in the presence of EIPA, activation of the NHE cannot account for the alkalinization.

To our current knowledge, no report has suggested an increase in Na-P_i type I cotransport activity as a cause of alkalinization at the onset of ATP depletion. However, the effect of Na-P_i cotransport on intracellular pH was recently studied. It was demonstrated that the influx of phosphate alkalinizes the cytosol of *Xenopus* oocytes expressing Na-P_i 3 (human type II) or Na-P_i 5 (flounder type II) (Moschén et al. 2001). Previous studies on the pH-dependence of Na-P_i cotransport showed that Na-P_i type II preferentially transports divalent phosphate (HPO_4^{2-}), whereas Na-P_i type III (the “housekeeping” Na-P_i cotransporter) preferentially transports monovalent phosphate: its transport rate is increased by extracellular acidification. Na-P_i type I when studied in oocytes seemed to have no preference for either monovalent or divalent phosphate (Kavanaugh and Kabat 1996; Moschén et al. 2001; Murer and Biber 1996). However, since $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ has a pK_a value of 6.8, the concentration of HPO_4^{2-} in the perfusion solution (pH 7.4) will be 4 times higher than the H_2PO_4^- concentration. Therefore, also the type I cotransporter is expected to alkalinize the cells by mainly transporting HPO_4^{2-} .

In 2001, Xiao and collaborators reported an increase in Na-P_i cotransport activity in rat proximal tubular cells subjected to ischemia-reperfusion injury. They observed that after 30 min of ischemia in freshly isolated brush border membrane (BBM) vesicles, Na-P_i cotransport activity had significantly increased by 31%, whereas Na^+ /glucose cotransport activity was decreased by 45%. The amounts of both Na-P_i type I and Na-P_i type II cotransporter protein were decreased after ischemia. Since Na-P_i type II is the predominant Na-P_i cotransporter in proximal cell brush borders, the authors attributed the increase in Na-P_i cotransport activity to Na-P_i type II (Xiao et al. 2001). Taking into account the present data, however, it is conceivable that an increase in Na-P_i type I cotransport activity was responsible for their observations.

Expression of Na-P_i type I in MTAL cells

The finding of Na-P_i cotransport activity in the apical membrane of rabbit MTAL cells was unexpected. In vitro perfused isolated rabbit TAL segments, there was no net transport of phosphate across the thick ascending limb (Rocha et al. 1977). Na-P_i 1 (i.e., rabbit type I) was shown to be present in the brush border of rabbit proximal tubules but absent in rabbit TAL, by mRNA detection (Custer et al. 1993) and immunohistochemistry (Biber et al. 1993). Whereas Na-P_i type II is responsible for phosphate reabsorption in the proximal tubule, the function and regulation of Na-P_i type I still is unclear (several reports have ruled out a prominent role for Na-P_i type I proteins in regulating body P_i homeostasis) (Werner et al. 1998). Cytosolic P_i plays a central role in cellular energy metabolism and in hormone-regulated glucose homeostasis. Li and collabora-

Fig. 9. Changes in pH_i during 60 min of metabolic inhibition with KCN (2.5 mmol/L) and deoxyglucose (DOG) (10 mmol/L). Na^+ was replaced by *N*-methyl-D-glucamine (NMDG $^+$) in all apical superfusion solutions and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) (10 $\mu\text{mol/L}$) was added to all apical solutions to prevent reverse operation of the apical Na^+/H^+ exchanger. Values are means $\pm$ SE, $n = 4$.

Fig. 10. Indirect immunofluorescence with (A) $\alpha\text{-Na-P}_i$ type I and (B) $\alpha\text{-Na-P}_i$ type II (dilution 1/200). (C) Control experiment in which the primary antibody was omitted.

tors (1996) demonstrated that in rat kidneys rNa-P_i 1 (rat type I) was upregulated by increasing plasma glucose levels, whereas Na-P_i 2 and Ram-1 (the “housekeeping” Na-P_i cotransporter) expression were unaffected. The authors did not investigate whether this rNa-P_i 1 expression was restricted to proximal tubular cells. This glucose-dependent increase in rNa-P_i 1 mRNA expression was independent of insulin, since it was observed in diabetic rats. In primary cell cultures from rat hepatocytes, high glucose concentrations (10–30 mmol/L) also increased rNa-P_i type I mRNA (but not Ram-1 mRNA) expression, but only if insulin (10 nmol/L) was present in the medium (Li et al. 1996). Since we have used a DME/F12 “high glucose” medium containing 25 mmol/L D-glucose, this might have induced the expression of Na-P_i 1 in these primary cell cultures of MTAL cells. Recently, molecular studies have determined that the SCLC17/type I phosphate transporters, a family of proteins initially characterized as phosphate carriers, mediate the transport of organic anions. While their role in phosphate transport remains uncertain, it is now clear that the transport of organic anions facilitated by this family of proteins is involved in diverse processes ranging from the vesicular stor-

age of the neurotransmitter glutamate to the degradation and metabolism of glycoproteins (Reimer and Edwards 2004).

Hypotheses on Na-P_i 1 activation during ischemia

A disadvantage of using DOG to block glycolysis is that a substantial fraction of total P_i is trapped as DOG-6-P (Allen et al. 1985). Therefore, although an increase in Na-P_i type I cotransport activity could possibly be related to the use of DOG, this hypothesis is unlikely since the alkaline transient occurs also in the presence of CN^- alone.

In 1985, Molitoris and collaborators (Molitoris et al. 1985) demonstrated that an increase in BBM Na-P_i cotransport activity could be mediated by a decrease in cholesterol content and an accompanying increase in membrane fluidity of the BBM. This might increase the transmembrane mobility of P_i transport units. In 1990, Friedlander and collaborators (Friedlander et al. 1990) investigated this further in primary cell cultures of rabbit proximal tubular cells. They were able to demonstrate that increasing the fluidity of the apical membranes (with the local anesthetic drug benzyl alcohol) decreased the Na^+ -dependent uptake of methyl α -D-glucopyranoside but increased the Na^+ -dependent P_i up-

take by 31%. Montagna et al. (1998) demonstrated in the rat "renal artery occlusion" model that after 50 min of ischemia, lipid peroxidation of proximal BBM had significantly increased, which may have caused increased membrane fluidity. Molitoris and Kinne (1987) have described that the reduction in Na⁺-dependent D-glucose transport (which is measurable after 15 min of renal artery clamping) correlated with ischemia-induced changes in membrane fluidity. Taking into account these data, the increased Na-P_i cotransport activity that was observed during ischemia could be the consequence of ischemia-induced modifications of membrane properties (Xiao et al. 2001).

In both rat kidneys and cultured hepatocytes, high glucose concentrations selectively upregulated Na-P_i type I expression (Li et al. 1996). In rat brain, BNP-1 (a Na-P_i cotransporter belonging to the type I Na-P_i cotransporter family) may be involved in brain energy metabolism (Ni et al. 1995). These data suggest that Na-P_i type I serves to supply the great demand of P_i in the liver, kidney, and brain as a result of their high level of glucose metabolism (Werner et al. 1998). Since ADP, AMP, and P_i are activators (or rather de-inhibitors of ATP) for phosphofructokinase (PFK), which is (at least in muscle) the major flux-controlling enzyme of glycolysis (Voet and Voet 1990), it is conceivable that a decrease in intracellular ATP content or an increase in ADP/ATP ratio might be the factor triggering Na-P_i type I activation.

The question arising from these experimental observations, in combination with the presented data from the literature, is whether an increase in Na-P_i type I cotransport activity is beneficial for cell survival. It has been demonstrated that incubation of OK cells (model of the proximal nephron) in phosphate-free medium for 2 h decreased intracellular ATP levels from 2.7 to 1.2 µg/mg protein, indicating the importance of adequate phosphate supply for cellular energy homeostasis (Green et al. 1993). In freshly isolated rat proximal tubules, phosphate-free perfusion during 40 min elicited cellular damage (Almeida et al. 1992). In neurons, P_i dose-dependently increases cell survival in the acute phase following oxidative insults (Glinn et al. 1998). These data suggest that an increased P_i uptake by ischemic cells might indeed be cytoprotective. Also, in a recent study comparing preservation solutions for kidney transplantation, it was demonstrated that a phosphate-buffered sucrose solution prevented tubular swelling more effectively than Euro-Collins or University of Wisconsin solutions, which contain substantially less phosphate (Ahmad et al. 2004).

In conclusion, metabolic inhibition produced a rapid and profound ATP depletion in primary cell cultures from rabbit MTAL. This ATP depletion was partially reversible after 1 h of MI. The changes in pH_i that were observed during MI were unexpected because a transient alkalization took place during the first phase of MI. It was demonstrated that this was not the result of either PCr breakdown or an increase in NHE activity, but rather the result of an increased activity of the Na-P_i type I cotransporter. Since the Na-P_i type I cotransporter is not expressed in MTAL cells in vivo, the importance of our finding for renal ischemia-reperfusion injury in vivo remains uncertain because it might be an artifact of the cell culture system used in this study.

Acknowledgements

We thank J. Janssen, W. Leyssens, P. Pirotte, and R. Van Werde for their technical assistance. We are grateful to H. Mürer and J. Biber (Institute of Physiology, University of Zürich, Switzerland) for kindly providing Na-P_i type I and II antibodies and to Prof. Dr. R.K.H. Kinne (Max-Planck-Institute für Molekulare Physiologie, Dortmund, Germany) for providing the TALH-SVE cell line. This work was supported by a grant from the Research Foundation Flanders (FWO GO270.07).

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