J. Membrane Biol. 128, 205–218 (1992)

The Journal of
Membrane Biology

Substrate-Induced Modulation of ATP Turnover in Dog and Rabbit Proximal Tubules

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Summary. In dog proximal tubules in suspension, the addition of glucose increased significantly the ouabain-sensitive fraction of respiration, a response suppressed by phlorizin. The addition of α-methyl-D-glucoside (α-MG) had a modest effect and 3-O-methyl-D-glucoside (3-O-MG) had no effect. The different stimulation of the Na^+,K^+-ATPase activity elicited for each hexose could be explained by a different increment of net transepithelial flux of sodium induced by the sodium : hexose cotransport. This flux is a direct function of the transport characteristics of both luminal and antiluminal membranes of proximal cells for these sugars: glucose is rapidly transported by both membranes (allowing a large transepithelial flux of glucose : sodium) while α-MG is poorly transported by the basolateral, and 3-O-MG by the luminal, membrane of the dog proximal tubule (allowing a small transepithelial flux of hexoses and sodium). However the overall tubular respiration of dog proximal tubules was not increased by glucose addition because the increment in the ouabain-sensitive fraction was accompanied by a reciprocal decrement in an ouabain-insensitive but oligomycin- or N′,N′ dicyclohexylcarbodiimide (DCCD)-sensitive (or in the bafilomycin-sensitive) component of respiration. This component reflects the activity of a large BBM-bound H^+-ATPase found in this species. The intracellular pH of dog proximal tubules in suspension was measured using the proton-sensitive fluorescent probe 2′,7′-bis-2-(carboxyethyl)-5, (and 6)-carboxyfluorescein. Glucose application significantly alkalinized the cells. In contrast, other substrates such as lactate or acetate simultaneously acidified the cells and increased the ouabain-insensitive phosphorylative respiration of dog tubules. These observations suggest that a modulation of the activities of both the sodium and most probably the proton pump is elicited by substrate availability in suspensions of proximal tubules.

Key Words hexose transport · intracellular pH · Na^+,K^+-ATPase · H^+-ATPase · bafilomycin A_1

Introduction

The renal reabsorption of many organic substances such as glucose, amino acids and organic acids involves sodium cotransport processes occurring at the brush-border membrane (BBM) of proximal tubules. A significant entry of sodium into proximal cells thus follows these reabsorptive processes, which secondarily stimulates the Na^+,K^+-ATPase activity. Suspensions of cortical tubules (mainly of proximal origin) represent a useful model to study the energetics of these processes in intact tubular cells. Such preparations have been used previously with success for this purpose [14, 26, 43]. Since proximal tubular segments present an open lumen [39] and since their BBM is exposed to a rather fixed concentration when exogenously added substrates are in the mM range, the reabsorptive processes occurring at the BBM proceed in vitro in a rapid fashion, i.e., the tubules may support a larger reabsorptive flux than in vivo. It is expected therefore that the fraction of the ATP turnover related to the Na^+,K^+-ATPase activity (measured as ouabain-sensitive respiration) should be significantly stimulated by an increment of these Na^+-dependent reabsorptive processes. The amplitude of the respiratory stimulation induced by substrate addition to proximal tubules should vary according to the type and concentration of substrates, the stoichiometry of cotransported species, the apparent Michaelis constant (K_m) of the transporter for the substrates and the amount of transporters present in the brush-border membrane. Furthermore, the mechanism of substrate efflux at the basolateral membrane will influence the net facilitated transport of sodium related to substrate traffic across the proximal cells [36, 39, 41]. Gullans, Harris and Mandel [14] have demonstrated a net and similar increment in respiration when glucose or its nonmetabolizable analog α-methyl-D-glucoside (α-MG) were added to a suspension of rabbit proximal tubules. This stimulation was entirely phlorizin-sensitive, indicating that it was indeed related to the cellular entry of these substrates through the hexose-Na^+ transporter localized in the BBM.

The present work was initiated by the observation that no stimulation of respiration was induced

by glucose addition to dog proximal tubules in contrast with the observation made by Gullans et al. [14] using rabbit proximal tubules. We have therefore examined the effect of the addition of various substrates including glucose, α-MG, 3-O-methyl-D-glucoside (3-O-MG), lactate and glutamine on phosphorylating and nonphosphorylating components of tubular respiration, on ATP content as well as on intracellular (pH_i) of proximal dog and rabbit tubular cells. Differences in the net rate of substrate : sodium cotransport explain the divergent observations made with glucose, α-MG or 3-O-MG on the ouabain-sensitive respiration in both species. Furthermore, a specific reduction of the ouabain-insensitive but oligomycin (or DCCD)-sensitive respiration was observed when glucose addition alkalinized the dog proximal cells. The reverse was observed with substrate-induced acidification of the cells. It is concluded that the overall effect of substrate availability on cell respiration is the result of complex interactions between different transport processes and should be analyzed in the light of each species context.

Materials and Methods

Preparation of Cortical Tubules in Suspension

The dogs were anesthetized with pentobarbital (30 mg/kg i.v.) and received an intravenous bolus of 20% mannitol (20 ml) before removing both kidneys which were immediately immersed in an ice-cold Krebs-Henseleit saline (KHS, containing (in mM): 120 NaCl, 3.2 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.5 $CaCl_2$, 25 $NaHCO_3$ and 50 mannitol). For studies with the rabbits, the animals were decapitated and both kidneys were immediately obtained. No mannitol injection was used in this species. The cortical tubules were prepared in an identical fashion for the two species as previously described for renal cortex of the dog [39] except that the concentration of calcium in the KHS was kept at 0.5 mM for the dog but increased to 2.5 mM for the rabbit to avoid dissociation of tubules. In both species, a final suspension containing around 60 mg wet wt/ml of cortical tubules was prepared and kept on ice. During the collagenase digestion of cortex slices, exogenous substrates were provided: 10 mM lactate + 1 mM pyruvate + 10 mM glucose for the rabbit and 10 mM lactate + 1 mM pyruvate + 10 mM glutamine + 1 mM glutamate (L + G) for the dog.

Determination of Enzyme Activities

The activity of critical enzymes was measured on homogenates of rabbit kidney cortex and compared to activities measured in homogenates of the dog cortex [31]. The homogenate was prepared as previously described, and the following activities were measured: γ-glutamyltranspeptidase (γ-GT) [38], alkaline phosphatase (AP) [46], Na^+,K^+-ATPase and membrane-bound oligomycin and vanadate-insensitive but NEM- or DCCD-sensitive H^+-ATPase [31]. Lactate dehydrogenase (LDH) activity was measured on homogenates of freshly prepared dog cortical tubules as well as on the supernatant obtained following a low-speed centrifugation of tubules incubated for different incubation times at 37°C [15].

Adenylate Measurement

The concentration of adenylates (ATP, ADP, AMP) was measured in tubules of both species incubated in the presence of the same substrates used in the digestion procedure and expressed in μmol/g wet wt. The effect of increasing concentrations of oligomycin on tissue ATP was evaluated.

The ATP concentration was also measured in tubules incubated without exogenous substrate, with 10 mM glucose, α-MG, L + G or a combination of these substrates in the presence or absence of 1 mM ouabain and 0.2 mM phlorizin. When required, the cell ATP was expressed as μmol/ml intracellular water using a factor of 0.55 μl of water/mg wet wt as determined from intracellular volume determination. The adenylates were measured by enzymatic assays as previously described [20, 23].

Metabolism Studies

The uptake of substrates (2 mM glucose, 2 mM lactate: 0.2 mM pyruvate) and the production of lactate from glucose by tubules of both species, incubated at 37°C in KHS equilibrated with 95% O_2 : 5% CO_2, were studied as a function of time. The synthesis of glucose from 2 mM lactate + 0.2 mM pyruvate or 2 mM glutamine + 0.2 mM glutamate was also estimated. The determination of glucose and lactate was as previously described [42].

Respiration Measurement

Aliquots of the tubule suspensions were preincubated for 5 min at 37°C in order to achieve thermic equilibrium before a sample (0.5 ml, ≈30 mg wet wt) was introduced in an oxymetric chamber of domestic design as previously described [39]. The consumption of oxygen by tubules was measured without or with one or a combination of the following substrates: 10 mM glucose, 10 mM α-MG, 25 mM 3-O-MG or 10 mM L + G. The subsequent addition of 2.5×10^{-7} M oligomycin was used to suppress 100% of the phosphorylating (ATP-synthetizing) component of cellular respiration (tissue ATP fell to zero, *see* Fig. 1). This phosphorylative component of respiration could be subdivided into two fractions: a ouabain-sensitive (1 mM ouabain) fraction reflecting the activity of the Na^+,K^+-ATPase, and a DCCD (oligomycin)-sensitive fraction (25 μM DCCD added after ouabain). DCCD inhibits all H^+-ATPases and therefore phosphorylation. The ouabain-insensitive but DCCD-sensitive fraction reflects the sum of all other tissue ATPases, including the membrane-bound H^+-ATPase [31]. The (ouabain + DCCD)-sensitive respiration equals the oligomycin-sensitive respiration. Since the two major ATPase activities of proximal tubules are Na^+,K^+-ATPase and H^+-ATPase, the ouabain-insensitive but DCCD- or oligomycin-sensitive respiration was taken as largely representing the activity of the H^+-ATPase *in situ* and will be referred to as "ouabain-insensitive phosphorylative respiration" throughout this paper.

In certain experiments, bafilomycin A_1, a specific inhibitor of vacuolar proton pumps [8] was used at the concentration of

5×10^{-7} M to inhibit the H^+-ATPase in presence of an intact Na^+,K^+-ATPase activity. This allowed to quantify in a different way the modulation of the proton pump activity induced by the substrate availability.

The effect of 0.2 mM phlorizin (Pz) (an inhibitor of the glucose- and α-MG-Na^+ cotransports) was studied to estimate the fraction of the OUA-sensitive respiration due to the Na^+ entry related to glucose or α-MG transport.

Intracellular pH Determination

The intracellular pH of dog proximal tubules in suspension (purified on a Percoll gradient according to the technique described by Vinay, Gougoux and Lemieux [42]) was measured using the BCECF-AM probe. The technique used to ensure a specific signal from intracellular BCECF was described in details elsewhere [33]. The pH_i was measured at 22°C in tubules suspended in KHS pH 7.4 (2.5 ml final volume) and kept under O_2 : CO_2 atmosphere, before and after addition of 10-mM substrates (50 μl of 500 mM glucose, α-MG, glutamine, lactate, pyruvate or acetate dissolved in K^+-supplemented saline: 4.5 mM KCl, 150 mM NaCl, adjusted to pH 7.4). For each measurement a calibration curve of pH_i was obtained following KCl-nigericin, NaOH or HCl addition, as proposed by Noël et al. [33] and Thomas et al. [40], in order to convert the fluorescence ratio $F500/F450$ into pH_i.

Hexose Transport Measurement in Brush-Border Membrane Vesicles

Brush-border membrane vesicles (BBMV) were prepared from homogenates [31] of dog and rabbit cortical tissue by magnesium precipitation using the previously described technique of Hopfer et al. [18] with the modifications introduced by Berteloot [7]. The membranes obtained from the dog cortex were fully characterized elsewhere [31].

The vesicles were loaded in a medium containing (in mM): 50 Tris-HEPES, 0.2 $MgSO_4$ and 120 NaCl or 120 KCl, adjusted to pH 6.0, 7.4 or 8.0. The extravesicular medium contained (in mM): 50 Tris-HEPES, 0.2 $MgSO_4$, 120 NaCl or 120 KCl and the pH was 6.0, 7.4 or 8.0 in order to establish or not a pH gradient across the membranes. Amiloride (0.5 mM) was added to inhibit the Na^+-H^+ exchanger which could potentially destroy or create a pH gradient. The uptake of [^{14}C]-glucose, [^{14}C]-α-MG and [^{14}C]-3-O-MG was studied using a concentration of 30 μM for each sugar. When indicated, 0.5 mM phlorizin (Pz) was added to inhibit the Na^+-dependent component of glucose uptake.

Uptake experiments were performed by incubating 20 μl (0.34 ± 0.05 mg protein (n = 3)) freshly prepared vesicles or frozen for less than 10 days for 0–7200 sec at room temperature in 80 μl of the above-mentioned medium containing the radiolabeled tracers. At the end of the incubation period, the medium was diluted in 2 ml ice-cold stop solution (50 mM Tris-HEPES, 120 mM KCl, and 0.5 mM phlorizin, pH 7.4) and rapidly filtered on 0.45-μm mesh Amicon™ filters. The filters were then washed four times with 2 ml stop solution, dissolved in Filter-Count™ (Packard) overnight, and counted.

Hexose Transport Measured in Cortical Basolateral Membrane Vesicles

Basolateral membrane vesicles (BLMV) of dog renal cortex were prepared according to a method derived from that of Hilden et al. [17], with the following modifications. About 30–40 g cortex slices were homogenized in 150–200 ml of buffer (250 mM sucrose, 50 mM Tris-HEPES, 0.1 mM phenylmethyl-sulfonylfluoride (PMSF), and 1 mM EDTA, pH 7.4) and centrifuged at 5,000 × g for 15 min in a Beckman JA-20 rotor. At this point, 10 mM $MgCl_2$ + 5 mM $CaCl_2$ + 2 mM $MnCl_2$ were added to the supernatant. The combination of these salts gave a better purification than $CaCl_2$ or $MgCl_2$ alone. The mixture was kept on ice for 10 min and continuously stirred before centrifuging in the same rotor at 2,000 × g for 10 min. This second supernatant was centrifuged at 30,000 × g for 20 min in a Beckman 60 Ti rotor. The membrane pellet was resuspended in 30 ml homogenizing buffer and re-extracted with the same combination of salts described above. After 10 min on ice, the mixture was again centrifuged at 2,000 × g for 10 min. The pellet was combined to that obtained following the first precipitation and resuspended in 30 ml of homogenizing buffer containing 20 mM EDTA to remove the cations. A centrifugation of 30 min at 30,000 × g was then performed. The resulting pellet was dispersed in 20 ml of resuspension medium (50 mM Tris-HEPES, 120 mM KCl and 0.2 mM $MgSO_4$, pH 7.4), and 4 ml of Percoll (Pharmacia Fine Chemicals) was added. The final Percoll concentration was 16%. Following a 30-min centrifugation at 30,000 × g, two distinct bands were separated. The upper tissue band (7 ml) was collected, mixed with 30 ml of the same buffer and centrifuged at 100,000 × g for 30 min in a 60 Ti rotor. This procedure was repeated twice to remove all traces of Percoll. The final pellet was resuspended in 1 ml of the same medium and passed 10 times through a 25½-gauge needle. The resulting preparation contained around 10 mg/ml protein of BLMV. The last washing procedure and the final resuspension can be made the following day without loss of hexose transport activities.

An enrichment factor of 8.8 ± 1.7 (n = 4) for the basolateral Na^+,K^+-ATPase and 1.7 ± 0.4 (n = 4) for AP were obtained. No significant Na^+-dependent lactate nor Na^+-dependent glucose transport was detectable in BLMV preparations (*not shown*), indicating that these vesicles were not contaminated by BBMV despite the presence of AP in significant amount. AP was already shown to comigrate with basolateral Na^+,K^+-ATPase in rat renal cortex [16].

The extravesicular medium was constituted of 50 mM Tris-HEPES, 0.2 mM $MgSO_4$, 120 mM KCl, adjusted to pH 7.4, and 30 μM of the radiolabeled tracers ([^{14}C]-glucose, [^{14}C]-α-MG or [^{14}C]-3-O-MG). Phloretin (Pt, 0.5 mM) was added to inhibit the sodium-independent basolateral transporter when required. Transport experiments were performed using 20 μl of BLMV (0.22 ± 0.04 mg protein (n = 3)) diluted in 80 μl of transport solution as described for BBMV. The stop solution contained 0.5 mM phloretin.

Materials

Ouabain, oligomycin, phloretin and phlorizin were purchased from Sigma. N,N'-dicyclohexylcarbodiimide (DCCD) was from Aldrich and amiloride hydrochloride was from Merck Frosst. Bafilomycin A_1 was provided to us by Prof. K. Altendorf, Osnabrück. Ouabain and amiloride were dissolved in H_2O, whereas DCCD, oligomycin, phloretin and phlorizin were dissolved in absolute EtOH. Bafilomycin A_1 was diluted in dimethylsulfoxyde. Acetoxymethyl form of 2',7'-bis-2-(carboxyethyl)-5, (and 6)-carboxyfluorescein (BCECF-AM) was provided by Molecular Probes. A stock solution made in ethanol was kept at −30°C, protected from light, for several months. Radiolabeled sugars were purchased from New England Nuclear. All other

Table 1. Metabolic and enzymatic characterization of dog and rabbit cortical tubules

	Rabbit	Dog
Metabolism studies (μmol · g wet wt^{-1} · h^{-1})		
Glucose utilization	33.7 ± 3.0 (4)	14.8 ± 1.4 (6)
Lactate production (Substrate: 2 mM glucose)	20.8 ± 2.7 (4)	1.7 ± 0.5 (7)
Lactate utilization (Substrate: 2mM lactate + 0.2 mM pyruvate)	39.1 ± 3.0 (4)	118.5 ± 14.5 (7)
Glucose synthesis		
(Substrate: 2 mM lactate + 0.2 mM pyruvate)	−3.3 ± 1.5 (4)	21.4 ± 0.9 (7)
(Substrate: 2 mM glutamine + 0.2 mM glutamate)	−5.7 ± 0.4 (4)	10.0 ± 0.7 (6)
Tissue adenylates (μmol/g wet wt)		
ATP	2.66 ± 0.11 (4)	1.96 ± 0.09 (19)
ADP	0.56 ± 0.07 (4)	0.52 ± 0.05 (13)
AMP	0.05 ± 0.02 (4)	0.09 ± 0.01 (13)
Enzyme activities (mU/mg protein)		
Renal cortex homogenate		
Alkaline phosphatase	171 ± 15 (4)	451 ± 82 (11)[a]
γ-glutamyltranspeptidase (γ-GT)	1096 ± 20 (4)	877 ± 60 (11)[a]
Na^+, K^+-ATPase	303 ± 57 (5)	579 ± 40 (11)[a]
H^+-ATPase	10 ± 5 (5)	80 ± 17 (11)[a]
Renal cortex BBMV		
H^+-ATPase (BBMV)	161 ± 21 (8)	576 ± 49 (11)[a]
γ-glutamyltranspeptidase	8789 ± 675 (8)	6132 ± 65 (11)[a]

[a] These data are taken from [31] and presented for the sake of comparison between both species.

chemicals used were of reagent grade and obtained from Fisher or Sigma. All enzymes were purchased from Boehringer Mannheim.

Statistical Analysis

All measurements are expressed as means ± SEM. When required, one-way ANOVA for repeated measurements were performed using the TADPOLE III software (Biosoft) and statistical significance of comparisons with a single control (no substrates) were obtained from the Studentized-Newman-Keuls test, considering a probability of $P < 0.05$ as significant.

Results

Metabolic and Enzyme Differences between Cortical Tubules Isolated from the Rabbit or the Dog Kidney Cortex

Table 1 compares metabolic characteristics of cortical tubules prepared from the kidney cortex of dog and rabbit. When tubules of both species were incubated with glucose, the rate of glucose oxidation was lower in dog than in rabbit tubules. In contrast, when lactate was used as substrate, dog tubules were able to extract a large amount of lactate while rabbit tubules were much less efficient. Significant gluconeogenesis from lactate or from glutamine alone was measured with dog tubules and at a faster rate with lactate than with glutamine. No glucose synthesis was observed with rabbit tubules in the conditions used here. However, it has been reported by us [47] as well as by Gullans et al. [13] and Zablocki and Bryla [48] that a net glucose synthesis can be observed in rabbit cortical tubules by using, respectively, α-ketoglutarate, lactate and alanine or α-ketoglutarate and alanine as starting substrates. Thus tubules of both species were metabolically active and the intracellular concentrations of ATP, ADP and AMP were comparable (Table 1). Each species studied demonstrated some metabolic specificity.

The activities of some critical enzymes are also presented on Table 1. The membrane-bound H^+-ATPase is more active in the dog than in the rabbit cortex despite the fact that the activity of the brush-

Table 2. Effect of substrate addition on the respiration of rabbit and dog cortical tubules

Substrates	Rabbit	Dog
No exogenous substrate	110.2 ± 5.4 (8)	185.2 ± 7.8 (13)
Glucose	139.2 ± 9.3 (8)*	182.2 ± 8.1 (13)
α-MG	109.7 ± 5.0 (8)	162.7 ± 8.8 (13)*
GLUC + α-MG	128.6 ± 12.1 (8)	180.5 ± 14.7 (13)
L+G	200.0 ± 5.6 (8)*	328.8 ± 14.8 (13)*
3-O-MG	NA	152.7 ± 12.5 (4)*

Data are expressed in μmol $O_2 \cdot g$ wet $wt^{-1} \cdot h^{-1}$ (means ± SEM (*n*)). Substrates are added at 10-mM concentration each: glucose (GLUC), α-methyl-D-glucoside (α-MG), lactate (L) and glutamine (G). 3-O-methyl-D-glucoside (3-O-MG) was added at 25-mM concentration.

* $P < 0.05$: comparison of the respiration observed with substrates to the no substrate situation in each species.

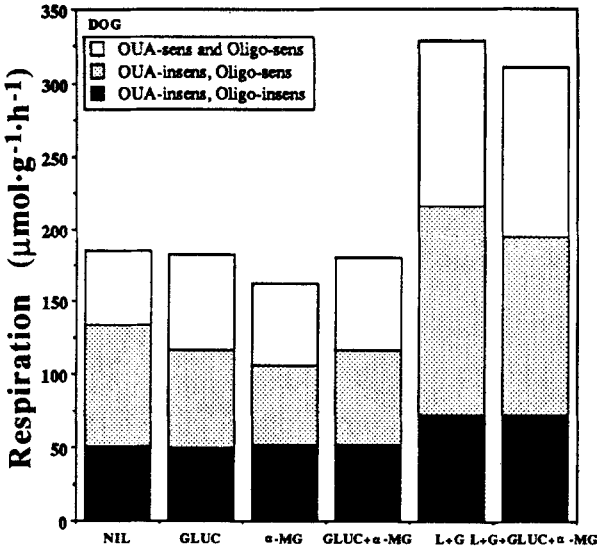

Fig. 1. Effect of substrate addition on the dog cortical tubule OUA-sensitive respiration (*OUA-sens*), the oligomycin-sensitive ouabain-insensitive respiration (*Oligo-sens, OUA-insens*) and the oligomycin-resistent respiration (*Oligo-insens*) compared to the nonsubstrate (*NIL*) situation. Abbreviations are defined in the legend of Table 2. Means of eight experiments.

border membrane marker γ-GT is larger in the rabbit than in the dog. Comparable activities of alkaline phosphatase and Na^+,K^+-ATPase were observed in cortex homogenates of these two species. Thus the ATP-dependent proton pumping activity may be a weaker mechanism in the rabbit than in the dog.

Effect of Substrate on Respiration of Cortical Tubules Isolated from the Dog and the Rabbit

Table 2 and Fig. 1 demonstrate that the addition of glucose, α-MG, or a combination of glucose and α-MG did not stimulate significantly the respiration of dog cortical tubules. Respiration remained close or below that observed with tubules incubated without exogenous substrates. In fact, a significant decrement was observed with α-MG addition. In contrast, the addition of lactate + glutamine increased significantly the overall respiration. In rabbit cortical tubules, glucose stimulated modestly the respiration while α-MG had no effect. The overall rate of respiration was lower in rabbit than in dog tubules.

The oxygen consumption related to the hydrolysis of ATP by the Na^+,K^+-ATPase can be estimated as the ouabain-inhibitable respiration since ouabain is a specific inhibitor of the sodium pump. Figures 1 and 2 show that the addition of glucose significantly increased the ouabain-sensitive respiration of dog tubules while α-MG (and 3-O-MG, *not shown*) was without effect. Moreover, the respiration induced by addition of glucose + α-MG was identical to glucose alone (*not shown*). L + G addition increased in a more marked fashion the oxygen uptake attributable to the Na^+,K^+-ATPase activity. Qualitatively similar observations were made with rabbit tubules.

In order to quantitate the cell ATP turnover in normal conditions, oligomycin, a specific inhibitor of the ATP synthetase, was used. The ATP content of dog cortical tubules falls to zero after addition of 20 μg oligomycin/g wet wt tubules which corresponds to 1.5×10^{-7} M in conditions used in this study (Fig. 3). This effect is rapid (within 2 min) and is accompanied with a large decrement in the respiration (phosphorylating respiration). Thus, this maneuver allows us to estimate the real ATP turnover of proximal cells defined as the oligomycin-sensitive respiration ×6, assuming a P/O ratio of 3. The ouabain-sensitive fraction represents only 55% of this respiration. The remainder of this phosphorylating respiration can be attributed to other ATP-hydrolyzing processes, representing at most 45% of total cell ATP turnover. Thus the oligomycin-sensitive but ouabain-insensitive respiration reflects all other ATP-hydrolyzing processes of the cells, including the activity of the H^+-ATPase.

A residual oligomycin-insensitive, nonphosphorylating respiration was always measured which represents about 30% of the total respiration. The addition of glucose, α-MG or glucose + α-MG did not change this oligomycin-insensitive nonphosphorylating mitochondrial respiration (Fig. 1). In contrast, lactate + glutamine addition increased this physiologically uncoupled component of respira-

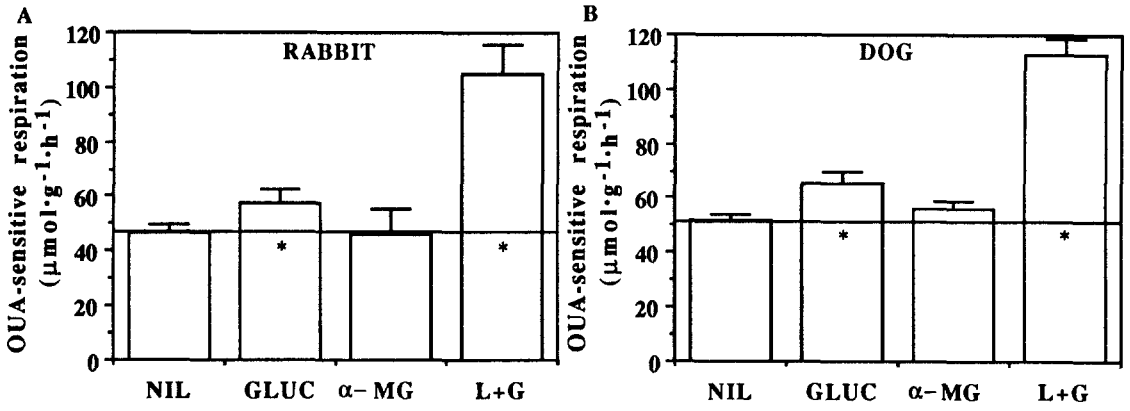

Fig. 2. Effect of substrate addition on the OUA-sensitive respiration of rabbit (*A*) and dog (*B*) cortical tubules. The horizontal line represents the mean respiration observed in the absence of exogenous substrates (*NIL*). Each substrate is added at 10-mM concentration. Data are means ± SEM, $n = 4$ (rabbit) and $n = 13$ (dog). $^*P < 0.05$; the statistical tests are described in Materials and Methods.

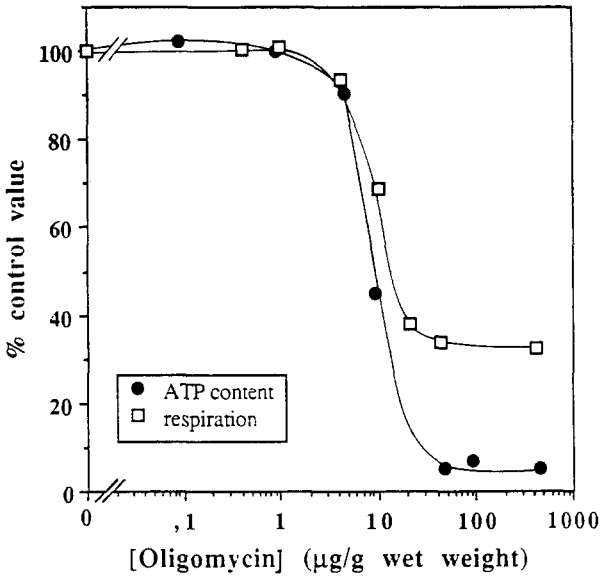

Fig. 3. Effect of oligomycin on ATP content and total respiration of dog cortical tubules in suspensions. Representative experiment.

tion. The subsequent addition of rotenone, an inhibitor of the aa_3 cytochrome, inhibited all oxygen uptake, demonstrating the mitochondrial origin of this oxygen consumption (*not shown*).

The fall of respiration induced by 2.5×10^{-7} M oligomycin following ouabain can be reproduced by 2.5×10^{-5} M DCCD. Figure 4 demonstrates that the addition of glucose, α-MG and L + G also significantly affected the DCCD (oligomycin)-sensitive but ouabain-insensitive component of respiration in dog tubules referred to as the ouabain-insensitive phosphorylative respiration. Indeed, glucose and α-MG significantly decreased this component of respiration while lactate + glutamine addition increased it. Again, the addition of glucose + α-MG had the same effect as addition of glucose alone (*not shown*). No significant inhibition of the small ouabain-insensitive phosphorylative respiration was observed upon glucose, α-MG or L + G addition in rabbit cortical tubules.

The specificity of the effect of glucose or α-MG on respiration was examined in dog tubules treated with phlorizin in order to inhibit the Na^+-dependent transporter which accepts these two substrates (Fig. 5). L + G was again used for comparison in order to exclude any nonspecific effect of phlorizin on phosphorylating respiration. Figure 5 demonstrates that preincubation of tubules with phlorizin completely inhibited the stimulation of the OUA-sensitive component of respiration induced by glucose or α-MG addition, while only a modest effect was noted upon lactate + glutamine addition. These results confirm the specificity of the effect of substrate addition on the ouabain-sensitive respiration. Similarly, the fall of the ouabain-insensitive phosphorylative respiration induced by glucose (and for lesser extent by α-MG) was completely prevented in presence of phlorizin. In contrast, the stimulation of this respiratory component observed with L + G was not significantly affected by the phlorizin treatment. Thus the changes of both ouabain-insensitive phosphorylative fraction of respiration induced by the addition of glucose or α-MG were mediated by the transport of these sugars through the phlorizin-sensitive Na^+ : glucose (α-MG) transporter.

Effect of Substrate Addition on the Intracellular Concentration of ATP

Figure 6 demonstrates that the intracellular concentration of ATP is unaffected by addition of glucose, α-MG or lactate + glutamine to tubules incubated

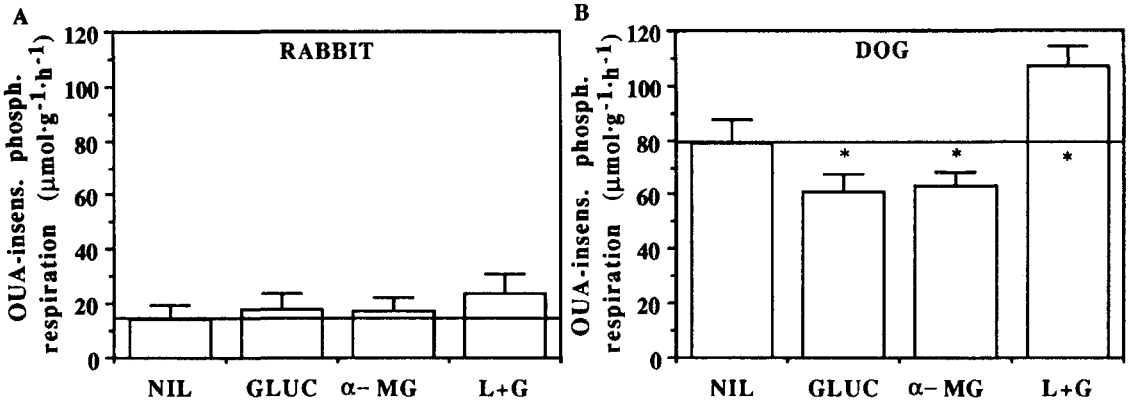

Fig. 4. Effect of substrate addition on the OUA-insensitive (*insens.*) phosphorylative (*phosph.*) respiration of rabbit (*A*) and dog (*B*) cortical tubules. *See* legend of Fig. 2 and Table 2 for details. $n = 4$ (rabbit) and $n = 13$ (dog). $^*P < 0.05$.

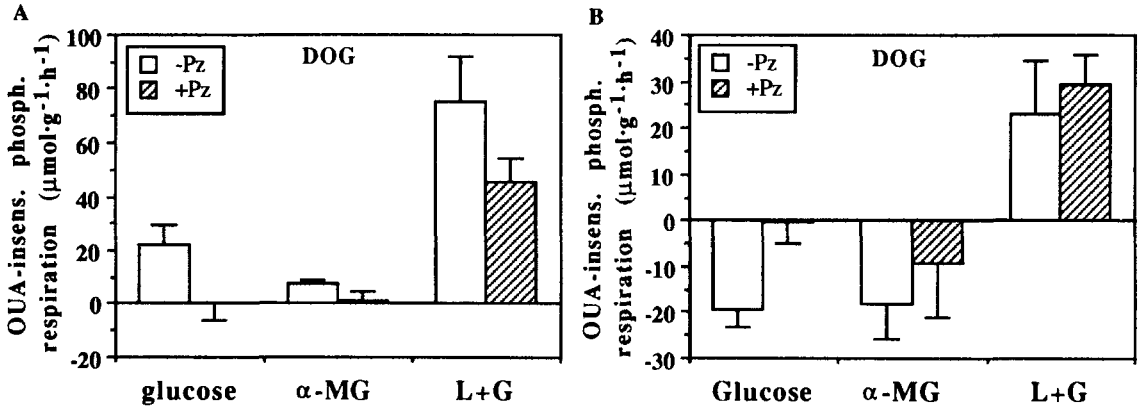

Fig. 5. Effect of incubation of dog cortical tubules with 0.2 mM phlorizin (Pz) on the OUA-sensitive (*A*) and on the OUA-insensitive phosphorylative respiration (*B*) induced by the addition of substrates to a suspension deprived of substrates. Values are means ± SEM ($n = 4$) of paired changes in OUA-sensitive or OUA-insensitive phosphorylative respiration induced by substrates compared to the nonsubstrate situation.

in the absence or in the presence of phlorizin or ouabain. Thus, the fall in the ouabain-insensitive phosphorylative respiration induced by glucose (and α-MG) addition as well as the stimulation of the Na^+,K^+-ATPase activity cannot be attributed to a modification of ATP concentration induced by these substrates. Note that in all these conditions the ATP concentration remains well above the apparent K_m for ATP of the main cellular ion-motive ATPases (Na^+,K^+-ATPase and H^+-ATPase; [31, 34]).

Intracellular pH in Dog Proximal Tubules

The intracellular pH (pH_i) of dog proximal tubules in suspension was measured before and after substrate addition in order to investigate if the changes in the ouabain-insensitive phosphorylative respiration (thought to be related to the H^+-ATPase activity) induced by substrate addition were mediated by changes in intracellular pH. The pH_i of proximal tubules in suspension studied under the control situation (no substrate added) was 7.37 ± 0.032 ($n = 6$ dogs, each individual value being the mean of 2–13 determinations) at an extracellular pH of 7.33 ± 0.021 ($n = 6$ dogs, 2–13 determinations). Figure 7 presents the effect of glucose, glutamine, lactate, pyruvate and acetate on pH_i. Glucose significantly alkalinized the cells, in a stable fashion, while lactate, acetate and pyruvate acidified them. α-MG alkalinized cells but only transiently. Glutamine had no significant effect on pH_i.

Figure 8 establishes that a significant correlation is observed between the changes in the ouabain-insensitive phosphorylative respiration of dog proximal tubules and the observed substrate-induced changes in the intracellular pH. This component of respiration is thus modulated by pH and may largely reflect the activity of the membrane-bound H^+-

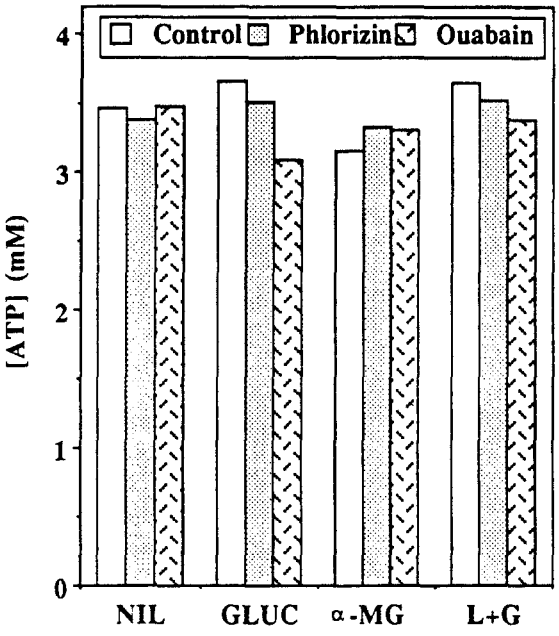

Fig. 6. Effect of incubation with different substrates on the ATP concentration ([ATP]) of dog cortical tubules incubated in the absence of effectors (control) and in the presence of phlorizin or ouabain.

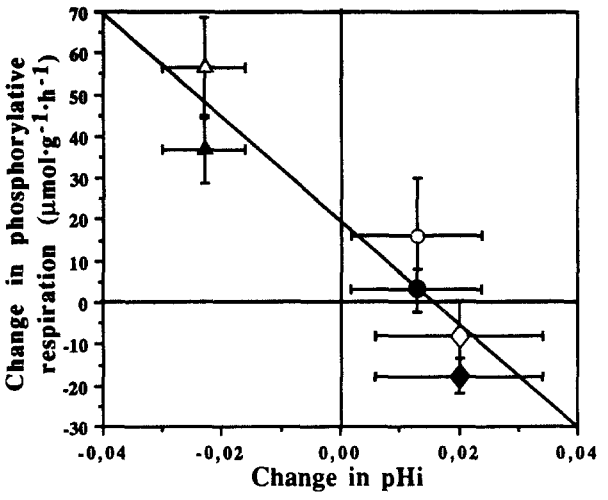

Fig. 8. Correlation between changes in ouabain-insensitive phosphorylative respiration (closed symbols) or bafilomycin-sensitive respiration (open symbols) and changes in pH_i induced by addition of 10 mM substrate to dog proximal tubules in suspension. Lactate (triangles), glutamine (circles), glucose (diamonds). For the ouabain-insensitive phosphorylative respiration, values were taken from series of data of Fig. 4 (n = 13) and Fig. 7 (n = 6), with their respective SEM. The regression curve is estimated from the mean points.

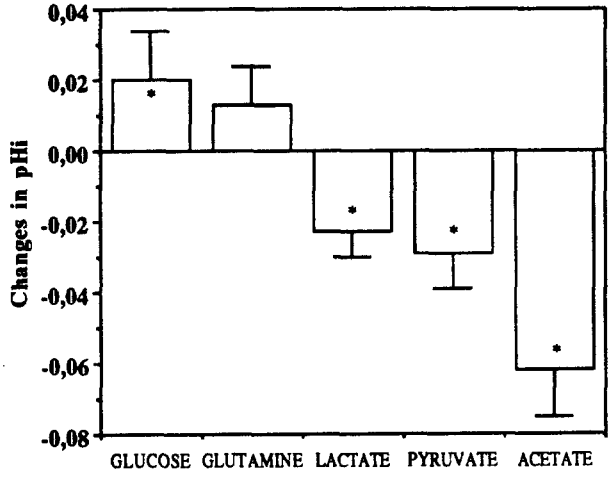

Fig. 7. Changes in intracellular pH (pH_i) induced by addition of 10 mM substrate to dog proximal tubules in suspension. The mean values presented on the figure are means of paired changes in pH_i measured upon substrate addition in six different tubule suspensions, each individual value being the mean of two to four determinations. $^*P < 0.05$; comparison with no change in pH_i.

ATPase. In another group of experiments, the effect of bafilomycin A_1 on tubule respiration was tested in the absence of ouabain and in the absence or presence of various substrates. Bafilomycin, a specific inhibitor of the proton pump with no effect on the Na^+,K^+-ATPase or mitochondrial H^+-ATPase at the concentration used, suppressed the phosphorylative respiration related to the proton pump. The effects of substrates on this component (Fig. 8, open symbols) of the cell ATP turnover was similar to that reported by using oligomycin or DCCD in the presence of ouabain (Fig. 8, closed symbols) (r^2 = 0.87).

Hexose Transport by Membrane Vesicles of Dog Kidney Cortex

The reason for different responses of respiration observed upon hexose addition was investigated by examining the transport of these sugars in luminal and in antiluminal membranes of dog proximal cells. When the transport of glucose and α-MG by BBMV was studied in the presence of a sodium gradient (120 mM NaCl out, 120 mM KCl in), a comparable accumulation of glucose and α-MG was observed with a significant overshoot reaching a maximum around 300 sec (Fig. 9). In the presence of a sodium gradient plus phlorizin (closed symbols) or in the absence of sodium (potassium being present in both the intra and extravesicular compartments; *not shown*), the glucose and α-MG uptakes were largely reduced and no overshoot was observed. These results demonstrate that both hexoses are transported

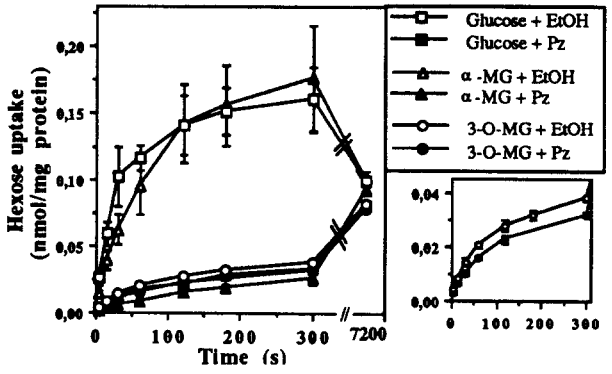

Fig. 9. Accumulation of glucose, α-MG and 3-O-MG in dog BBMV measured in the presence of an inwardly directed Na^+-gradient (120 mM out; 0 in) in the absence (EtOH) and in the presence of phlorizin (*Pz*), as a function of time. Accumulation measured at equilibrium (7200 sec) is indicated. For more clarity, the 0–300 sec uptake of 3-O-MG measured in the absence and in the presence of *Pz* is shown in the insert at the right. Data are means ± SEM, $n = 3$ experiments.

by BBMV isolated from the dog cortex in a similar fashion, a process involving a Na^+-dependent, phlorizin-sensitive mechanism. These results do not explain the different effect of both sugars on dog tubule respiration.

In contrast, a very small Na^+-dependent tracer accumulation was observed with 3-O-MG (Fig. 9). These observations explain the lack of stimulation of Na^+,K^+-ATPase activity observed in presence of 3-O-MG (no entry of Na^+ into cells).

Transport of glucose, α-MG and 3-O-MG was also evaluated on dog cortical BLMV to study the facilitated Na^+-independent glucose transport in these membranes. Figure 10 shows that no significant phloretin-sensitive component of α-MG accumulation could be detected in BLMV while a large phloretin-sensitive glucose and 3-O-MG uptake was found. Thus a difference in the efflux of hexoses out of proximal cells may explain the different effects observed with glucose, α-MG or 3-O-MG on respiration.

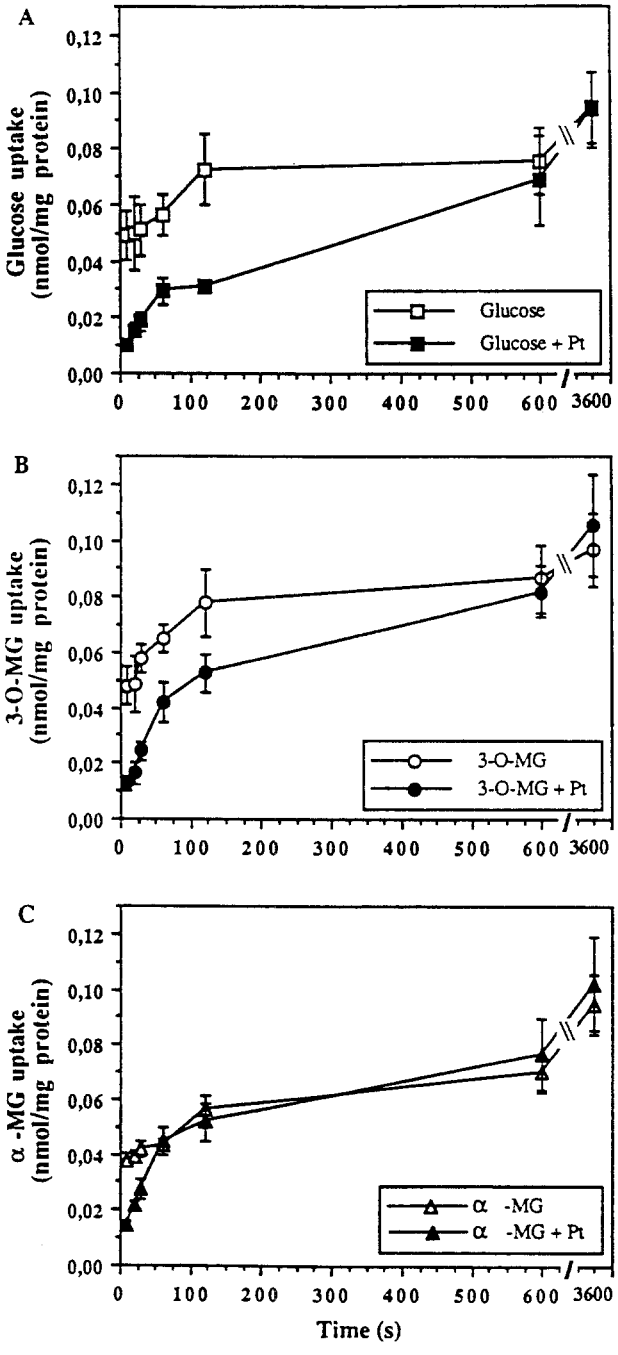

Fig. 10. Accumulation of glucose (*A*), 3-O-MG (*B*) and α-MG (*C*) in dog BLMV measured in KCl medium in the absence and the presence of phloretin (*Pt*), as a function of time. Accumulation of tracer measured at equilibrium (3600 sec) is indicated. Data are means ± SEM, $n = 3$ experiments.

Discussion

Cellular Integrity

In intact cells in vitro, the study of substrate transport and metabolism requires a cellular membrane integrity allowing compartmentation and maintenance of ions and metabolite gradients across the plasma membrane. We thus evaluated the tubular integrity along the incubation procedures by measuring the activity of LDH released into the medium as a function of time. After 60-min incubation, this activity reaches 10.3 ± 1.4% ($n = 7$) of the activity measured in homogenates of freshly prepared tubules [1]. This demonstrates a minimal cellular mem-

brane destruction. In accord, these tubules excluded well the dye Trypan blue and no stimulation of respiration was observed upon ADP addition, excluding the possibility that functional mitochondria were directly exposed to exogenous ADP and other substrates. Large sodium and potassium gradients are created by dog proximal tubules maintained under agitation in KHS: these cells maintain a low intracellular sodium concentration, around 20 mM, and a high intracellular potassium concentration [2, 39]. In accord, a large increment in intracellular sodium concentration is induced upon addition of ouabain [2, 3] or nystatin and a large stimulation of ouabain-sensitive respiration is observed upon addition of nystatin [3, 39]. These observations clearly indicate that dog proximal tubules in suspensions are capable of active sodium transport and that the Na^+,K^+-ATPase is not normally saturated by sodium. All together, these results indicate that the cellular integrity of our tubule preparation is well maintained.

Transepithelial Flux of Substrates

The observations reported here demonstrate that in dog cortical tubules, the supply of glucose increases the fraction of cellular ATP turnover specifically related to the Na^+,K^+-ATPase activity. This is a functional consequence of the entry of sodium together with this hexose through the specific sodium cotransporter system localized in the brush-border membrane. Indeed, the addition of phlorizin which specifically inhibits the glucose : Na^+ cotransport fully prevented this stimulation of respiration. These observations were expected and duplicated previous observations made in rabbit proximal tubules by Gullans et al. [14].

In contrast and despite the fact that the BBM transports α-MG and glucose similarly (vesicle data), no significant stimulation of the ATP turnover related to the activity of the Na^+,K^+-ATPase was observed upon addition of α-MG. Substances entering the luminal membrane of proximal tubular cells together with Na^+ in a similar fashion should stimulate equally the Na^+,K^+-ATPase activity and thus induce a comparable increment in ouabain-sensitive respiration. The quantitatively different metabolic effects induced by glucose and α-MG addition on the ouabain-sensitive ATP turnover of dog cortical tubules are probably related to differences in sodium entry created upon addition of these sugars. The amplitude of the stimulation of the Na^+-dependent respiration elicited by substrate addition should vary, however, according to the type of the cotransported species. Indeed, the stoichiometry of the Na^+ : substrate transport process, the affinity of the substrate for the transporter, the turnover and the number of transporter units in the membrane may all affect the net influx of sodium through the brush-border membrane. Moreover, the mode of exit of the transported species at the basolateral membrane (affinity of the transporter for the substrate, number of transporters) will also regulate the net transepithelial flux of these substances: a rapid basolateral exit facilitating (no cellular accumulation) and a slow exit inhibiting (cellular accumulation) the cell entry of both Na^+ and the cotransported species. Thus, the magnitude of the ouabain-sensitive stimulation of respiration induced by substrate addition should be determined by the transport characteristics of both brush-border and basolateral membranes of proximal cells.

The fact that glucose induced a greater stimulation of Na^+,K^+-ATPase than α-MG can be analyzed in the light of these considerations. Both sugars enter the BBM through the same Na^+-dependent transporter, which present in the dog a similar affinity for these sugars when studied on whole kidney [36] and comparable apparent kinetics parameters when studied in brush-border membrane vesicles [37]. However, the basolateral membrane transport (and thus the exit out of cells) proceeds at a much faster rate for glucose than for α-MG. This was shown here for the dog BLMV and also by Mullin, Fluk and Kleinzeller [29] and by Ullrich and Papavassiliou [41] for other species. The OH group in position C_1 of the pyranose ring necessary for optimal interaction of hexose with the phloretin-sensitive glucose transporter is lacking on α-MG [36]. A slower basolateral exit of α-MG could thus lead to a faster and larger intracellular accumulation of α-MG that would secondarily inhibit its apical entry into proximal cells, by creating an unfavorable gradient of concentration. A secondary backflux of α-MG into the lumen [36] through the Na^+-dependent glucose transporter could also result in a reduced net entry of Na^+ into cells. According to both schemes, the net luminal entry of substrates and Na^+ and the secondary stimulation of Na^+,K^+-ATPase is expected to be less for α-MG than for glucose in dog cortical tubules. These considerations can account for the different effect observed in intact cortical tubules isolated from the dog cortex upon application of these sugars.

Similar experiments were not performed with rabbit basolateral membranes in this study since these informations were already available: α-MG is also poorly transported by rabbit BLMV. Indeed, Cheung and Hammerman [9, 10] observed a transtimulation of ^{14}C-D-glucose uptake in rabbit BLMV by D-glucose, 2-deoxyglucose and 3-O-MG but not

with α-MG. Our measurements of substrate-induced stimulation of ouabain-sensitive respiration in rabbit tubules also suggest a smaller transepithelial flux of α-MG than glucose as observed in dog tubules.

No stimulation of Na^+,K^+-ATPase was observed upon addition of 3-O-MG to the suspension of cortical tubules. This was expected since the luminal entry of this substrate with Na^+ is small. Indeed, the affinity of the dog BBM glucose transporter for 3-O-MG is very low: Silverman [36] demonstrated that almost 100% of delivered 3-O-MG is recovered in urine in experiments performed on whole dog kidney. Furthermore, a very high concentration of 3-O-MG is necessary to inhibit the glucose uptake in dog BBMV [37], human fetal jejunum BBMV [25] or the α-MG uptake in oocytes [19]. A poor transport of 3-O-MG was also shown by Amsler and Cook [4] in LLC-PK_1 cells. It has also been found in LLC-PK_1 cells that the $K_{1/2}$ to stimulate the short-current circuit with 3-O-MG is 29 mM [28]. Therefore, it is not surprising that no effect on the ouabain-sensitive respiration was observed when presenting 25 mM 3-O-MG to dog tubules, i.e., the conditions used in the experiments presented here. A 100–300 mM 3-O-MG concentration would be expected to produce the same effect as glucose on ouabain-sensitive respiration, since the efflux pathway at the basolateral membrane is not limiting for this sugar in the dog. This maneuver is not practicable when working on intact tubules under physiological osmolality.

Therefore, a variable stimulation in the activity of the sodium pump should occur upon addition of each hexose. A net stimulation of the overall ATP turnover is thus expected. However, this was not observed, and the reason for this apparent discrepancy is now discussed.

Substrate-Induced Changes in pH_i

The addition of glucose or α-MG did not stimulate or even inhibit (α-MG) total tubular respiration in the dog. Indeed, the availability of glucose and α-MG simultaneously suppressed the ouabain-insensitive phosphorylative component of respiration. This fraction of the ATP turnover includes the functional expression of the H^+-ATPase activity functioning as a proton pump in the dog BBM [31, 43]. Considering that (i) the NEM- or DCCD-sensitive membrane-bound H^+-ATPase is a major ATPase of canine proximal tubule, (ii) the endosomal H^+-ATPases are probably not optimally active at physiological intracellular pH [11, 21], and (iii) the work performed by Ca^{2+}-ATPases should be low compared to secretion of protons into the lumen, we propose that the DCCD (oligomycin)-sensitive ATP turnover largely reflects the activity of the membrane-bound H^+-ATPase in this nephron segment.

The decrement in ouabain-insensitive phosphorylative respiration observed upon addition of glucose was related to glucose transport since the addition of phlorizin fully prevented this effect. Because the stimulation of respiration related to Na^+,K^+-ATPase (ouabain-sensitive respiration) and the suppression of the ouabain-insensitive phosphorylative respiration induced by glucose addition were reciprocal phenomena of the same magnitude in dog tubules, no net effect was observed on total respiration, in contrast to the observations reported by Gullans et al. [14] with rabbit proximal tubules. In this latter species, this reciprocal phenomenon could not be demonstrated by Gullans nor by us presumably because both the ouabain-insensitive phosphorylative respiration and the H^+-ATPase activity in BBM are small in the rabbit. H^+-ATPase activity was found to be three- to fourfold lower in BBM isolated from rabbit cortex than in dog BBM. In intact tubules isolated from the rabbit cortex, the ouabain-insensitive ATP turnover was also three- to fourfold lower than in dog tubules.

No inhibition of respiration was observed after adding DCCD (similar DCCD/tissue ratio) to ouabain-treated dog thick ascending limbs, a tissue rich in mitochondria and in Ca^{2+}-ATPase activity [39] but mostly devoid of membrane-bound H^+-ATPase activity [45]. Thus the ouabain-insensitive, but DCCD (oligomycin)-sensitive component of respiration is specific for proximal tubules. We consider that this is another argument in favor of the hypothesis that the ouabain-insensitive phosphorylating component largely reflects the ATP turnover attributable to the activity of the brush-border membrane-bound proton pump [31].

Assuming that this component of respiration largely reflects the activity of the membrane-bound proton pump, this would indicate that active H^+ pumping by proximal tubules in vitro is not as large an ATP-requiring process in the rabbit as in the dog. Thus, no measurable suppression of this activity could be observed in rabbit proximal tubules by us, nor by Gullans et al. [14] when hexoses were added, and no significant stimulation of this activity was found when lactate + glutamine were provided.

It should be noted that neither the synthesis of ATP occurring at substrate level nor the Crabtree effect [22] can explain the decrement in the ouabain-insensitive phosphorylative respiration observed in dog cortical tubules upon glucose addition since α-MG, a nonmetabolizable hexose, duplicates this effect.

In intact dog tubules, pH_i measurements demonstrated that glucose induced a sustained intracellular

alkalinization. Some substrates are known to enter cells with H^+ or through exchange with OH^- [12] or HCO_3^- [45] and could potentially induce a change in intracellular pH. Such a change could stimulate or inhibit the H^+ transport mechanisms responsive to pH ($Na^+ : H^+$ exchanger or H^+-ATPase) [31]. To examine if glucose transport could directly induce a change in pH_i, transport experiments were performed on BBMV exposed to various pH gradients. Glucose accumulation was not modified by acidic or alkaline intravesicular pH. Thus, glucose transport *per se* is unlikely to be accompanied by a simultaneous H^+ translocation out of BBM of the dog proximal tubules, in accord with the known characteristics of the various renal glucose carriers.

A cellular alkalinization induced by the inhibition of HCO_3^- exit secondary to the cellular depolarization produced by the large electrogenic entry of Na^+ : glucose may be postulated to explain our observations. In agreement with this view, it has been shown that the addition of phenylalanine, a sodium-cotransported amino acid, to proximal convoluted tubule cells of the frog kidney also induced a peritubular membrane depolarization accompanied by an increase in pH_i [27]. Glucose was also shown to induce alkalinization of rabbit microperfused proximal convoluted tubules [6, 24] as well as a cellular depolarization [24]. Such alkalinization may in turn suppress the H^+-ATPase activity and, therefore, the ouabain-insensitive phosphorylative respiration in species presenting a pH-responsive modulation of the H^+-ATPase activity [31, 32]. This was clearly the case with dog tubules.

In contrast, the transitory cellular acidification observed upon lactate, acetate or pyruvate addition could be attributed to the entry of these substrates with a H^+ through a nonionic diffusion [30] and/or through an exchange with cellular OH^- (HCO_3^-) [12]. Recent observations of our laboratory demonstrated that an uptake of ^{14}C-lactate above the equilibrium is driven in BBMV and also in BLMV of dog proximal tubules by the establishment of a H^+ gradient, but not a HCO_3^- gradient, across the membrane (acidic outside) [44]. This entry of H^+ may explain in part the stimulation of the ouabain-insensitive phosphorylative respiration observed with lactate, pyruvate, and acetate addition in dog proximal tubules. Thus, it appears that the ouabain-insensitive ATP turnover of proximal tubules in vitro is responsive to small substrate-induced variations of pH_i. Comparable responses to pH manipulations were directly demonstrated for the H^+-ATPase and the proton pump of isolated BBM [31]. The negative (glucose and α-MG) and positive (L + G) modulations of this activity by small pH changes suggest that this ouabain-insensitive phosphorylative respiration is of physiological importance. Variations of pH_i could modulate the H^+-ATPase by a direct protonation of a regulatory site, as it was demonstrated for the $Na^+ : H^+$ exchanger [5], by stimulating the turnover of pumps in the membrane, by inducing exocytosis of endosomal proton pumps [35], by increasing the availability of H^+ to be transported or by other mechanisms such as altering membrane fluidity, changes in membrane potential, and changes in intracellular calcium concentration.

Our experiments with bafilomycin added directly to a suspension of tubules exposed to various substrates (in absence of ouabain) confirm that the substrate-induced modification of the ouabain-insensitive phosphorylative respiration is indeed due to a modulation of the activity of the H^+-ATPase. These results thus rule out possible effects of DCCD on the $Na^+ : H^+$ exchanger activity or Na^+-coupled transport as an explanation for our findings.

We have presented evidence in this paper that glucose entry induces a stimulation of Na^+,K^+-ATPase activity in cortical tubules and that it simultaneously reduces the ouabain-insensitive phosphorylative component of respiration. This effect is not due to a depletion of cellular ATP or to a stimulation of glucose metabolism. We have presented evidence that the effect of glucose on the ouabain-insensitive phosphorylative respiration could be related to changes in pH_i. The results presented also show that the maximal ATP turnover related to the activity of the H^+-ATPase may be as large as that required for the activity of the Na^+,K^+-ATPase in dog proximal tubules in suspension and is three times larger in dog than in rabbit proximal tubules. We propose that the ATPase responding to pH changes is the BBM-bound H^+-ATPase, sensitive to bafilomycin A_1. In accord, the change in respiration observed upon changes of pH_i is large (high H^+-ATPase activity) in the dog proximal tubules but small in the rabbit (low H^+-ATPase activity).

The overall effect of substrate availability on cell respiration is thus the result of complex interactions between different transport processes and cannot be attributed to a single event unless thoroughly demonstrated.

This work was presented in part at the 22nd and the 23rd annual meeting of the American Society of Nephrology in Washington in December 1989 and 1990. The authors are grateful to Dr. Andrew Baines and Dr. Alfred Berteloot for fruitful discussions. The technical assistance of Jacques Sénécal is acknowledged. This work was supported by the Medical Research Council of Canada (grant M-7875). J. Noël received a MRC studentship.

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Received 2 October 1991; revised 4 February 1992