Type II Na-P_i cotransport is regulated transcriptionally by ambient bicarbonate/carbon dioxide tension in OK cells

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[1]*Institute of Physiology, University of Zurich-Irchel, CH-8057 Zurich, Switzerland;* [2]*Department of Medicine, Division of Nephrology, University of Louisville, Louisville, Kentucky 40292; and* [3]*Klinik B für Innere Medizin, Kantonsspital, CH-9007 St. Gallen, Switzerland*

Jehle, Andreas W., Helene Hilfiker, Markus F. Pfister, Jürg Biber, Eleanor Lederer, Reto Krapf, and Heini Murer. Type II Na-P_i cotransport is regulated transcriptionally by ambient bicarbonate/carbon dioxide tension in OK cells. *Am. J. Physiol.* 276 (*Renal Physiol.* 45): F46–F53, 1999.—The purpose of the present study was to determine whether isohydric changes in HCO_3 concentration and P_{CO_2} directly affect apical Na-dependent P_i (Na-P_i) cotransport in OK cells (opossum kidney cell line). Cells were kept at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (high-HCO_3/CO_2 condition), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (low-HCO_3/CO_2 condition) (for 14–24 h). Incubation in lower HCO_3/CO_2 concentrations increased Na-P_i cotransport 1.5-fold. The increased Na-P_i cotransport was paralleled by a two- to threefold increased expression of the NaPi-4 transporter protein and a two- to threefold increase in NaPi-4 mRNA abundance. The increase in NaPi-4 mRNA could be completely prevented by incubation in the presence of a transcriptional inhibitor, suggesting that the increase in NaPi-4 mRNA results from an increased NaPi-4 mRNA transcription. In agreement, the NaPi-4 promoter activity was stimulated by 50% at lower HCO_3/CO_2 concentrations. In conclusion, our data demonstrate that isohydric changes in HCO_3 concentration and P_{CO_2} exert a significant, direct cellular effect on Na-P_i cotransport and NaPi-4 protein expression in OK cells by affecting NaPi-4 mRNA transcription.

acidosis; alkalosis; parathyroid hormone; promoter; proximal tubule

THE INTERRELATIONSHIPS between acid-base and phosphate metabolism are manifold and complex. Acid-base disturbances have important effects on the renal handling of phosphate, whereas alterations in phosphate balance affect systemic acid-base homeostasis. Administration of phosphate has been demonstrated to induce metabolic alkalosis by increasing renal bicarbonate retention both in rats and humans (9, 15), whereas phosphate depletion induces metabolic acidosis by impairing renal proton secretory capacity (5).

Renal phosphate excretion is increased in chronic metabolic acidosis in humans resulting in renal hypophosphatemia (17). Metabolic alkalosis induced by bicarbonate infusion was also shown to increase renal phosphate excretion in parathyroidectomized rats (31). Respiratory acid-base disturbances also affect renal phosphate handling. Acute respiratory acidosis increased renal P_i excretion in rats (30), whereas chronic respiratory alkalosis was demonstrated to result in increased phosphate reabsorption and renal hyperphosphatemia in humans (16, 20).

Information on the mechanisms of regulation of renal phosphate transport by changes in acid-base balance is of great physiological and pathophysiological interest for at least two reasons: *1*) alterations in renal phosphate transport by pH, HCO_3, and/or CO_2 concentrations are expected to (co)determine the renal systemic response to acid and alkali loads, that is, to affect the severity of acidemia or alkalemia; and *2*) alterations in renal phosphate transport induced by changes in acid-base homeostasis change phosphate stores and, thereby, might affect multiple systemic processes and functions, including bone mineralization and functions of erythrocytes, leukocytes, and myocardium (12).

We have shown previously in opossum kidney (OK) cells that a proximal tubule-specific Na-dependent P_i transporter (Na-P_i cotransporter) is stimulated by low pH and low HCO_3 concentrations by posttranscriptional mechanisms. This stimulation of the Na-P_i cotransport was prevented by dexamethasone (10), suggesting that the phosphaturia typical of metabolic acidosis might not be a direct consequence of acidemia but, at least in part, of associated systemic effects (enhanced glucocorticoid activity; Refs. 2 and 10). In addition, in vivo acid-base disturbances result in rather modest changes in hydrogen ion concentration due to compensatory mechanisms, which raises some doubts on the quantitative effects of increases in hydrogen ion concentration in vivo on Na-P_i cotransport activity.

The goals of the present studies were to evaluate whether Na-P_i cotransport is regulated by HCO_3/CO_2 independent of hydrogen ion concentration (isohydric environment). Previous studies on the regulation of intracellular pH in renal cells demonstrated that cell pH varies in direct linear proportion with extracellular hydrogen ion concentration (26). Only changes in extracellular pH (by changing HCO_3 or CO_2) affect intracellular pH significantly, and isohydric changes in HCO_3/CO_2 were shown to exert no significant effect on cell pH (14). Therefore, it appears that isohydric changes of HCO_3/CO_2 concentrations affect the cell and the regulation of its functions differently than do changes of extracellular pH (by changing HCO_3 or CO_2). For the present study, we used opossum kidney cells (OK cells, Ref. 13), which represent a well-characterized model with which to study the regulation of proximal tubular Na-P_i cotransport (1, 3, 4, 19, 23) and which contain an

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apically located type II Na-P_i cotransporter (NaPi-4, Ref. 5).

MATERIALS AND METHODS

Cell cultures. All cell culture supplies were obtained from GIBCO-BRL (Basel, Switzerland). Opossum kidney cells (OK cells, clone 3B/2) were grown in DME + Hams's F-12 medium (DME-F12, 1:1) supplemented with 10% FCS, 22 mM $NaHCO_3$, 20 mM HEPES, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C.

For experiments, cells were seeded on 35-mm petri dishes (Nunc) or 10-cm petri dishes (Corning) and grown to confluence in the media indicated above. Subsequently, cells were deprived of serum for 24 h in DME-F12 medium (1:1) supplemented with 44 mM $NaHCO_3$, 50 IU/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 10% CO_2-90% air at 37°C. The pH was controlled with a microelectrode and adjusted to pH 7.4 with $NaHCO_3$ or HCl. For experimental incubations (time interval as given in RESULTS), the cells were kept in DME-F12 medium (1:1) supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin and with 44 mM $NaHCO_3$, pH 7.4, in a humidified atmosphere of 10% CO_2-90% air at 37°C (control) or with 22 mM $NaHCO_3$, pH 7.4, and additional 22 mM NaCl (for equalization of the osmolality difference) in a humidified atmosphere of 5% CO_2-95% air at 37°C (experimental). Again, the pH was controlled with a microelectrode and adjusted to pH 7.4 for the control and experimental condition with $NaHCO_3$ or HCl. Additionally, the pH was controlled a number of times at the end of the incubation periods. Even after prolonged incubation periods, the pH was never more than 0.04–0.06 pH units smaller than at the beginning of the experiments.

Membrane preparation. Cells grown in 10-cm petri dishes were washed with cold 0.9% NaCl and 10 mM Tris·HCl [pH 7.4, Tris-buffered saline (TBS)] and subsequently with 5 mM HEPES-KOH (pH 7.4). A volume of 15 ml of 5 mM HEPES-KOH supplemented with 4 mM EDTA and with the protease inhibitor phenylmethylsulfonyl fluoride (1 mM, Sigma) was added, and the cells were scraped off the dish. For homogenization, the cell suspension was passed five times through a 20-ml syringe connected to a 20-gauge needle. The homogenized suspension was centrifuged at 31,000 rpm for 40 min at 4°C (Sorvall ultracentrifuge OTD 50B, T 865 rotor). The pellet was resuspended in 400 μl of 50 mM mannitol with 10 mM HEPES-Tris (pH 7.2). The protein concentration was determined by the Bio-Rad protein assay.

SDS-PAGE and immunoblotting. Equal amounts of the membrane preparations were used for the SDS-PAGE (9% gels) (18). The separated proteins were transferred onto cellulose-nitrate (BA 83, Schleicher & Schuell) according to Towbin et al. (27). Nonspecific binding was blocked by incubating the nitrocellulose in TBS containing 5% nonfat dry milk and 1% Triton X-100 (Blotto-TX-100) at room temperature for 2 h. The NaPi-4 protein was detected using a polyclonal antiserum raised against the COOH-terminal 12 amino acids of the published NaPi-4 sequence (antibody dilution 1:2,000) (25). For antibody production (in New Zealand White rabbits), the NaPi-4 peptide was coupled to keyhole limpet hemocyanin. The specificity of this antiserum was previously established by a peptide protection assay (10). Incubation with the primary antibody took place overnight at 4°C. The nitrocellulose was washed four times with TBS + 10% Blotto-TX-100 and incubated for 1 h with Blotto-TX-100 at room temperature. Thereafter, the nitrocellulose was incubated with a 1:10,000 dilution of an anti-rabbit immunoglobulin labeled with horseradish peroxidase (Amersham) in Blotto-TX-100 for 2 h at room temperature. The nitrocellulose was washed four times with TBS. The signals were detected by the enhanced chemiluminescence system (Amersham) according to the manufacturer's protocol using Kodak X-OMAT AR films, and they were quantified by densitometry. Broad-range SDS-PAGE molecular protein markers (Bio-Rad) were run in parallel.

RNA isolation, Northern blot analysis, and densitometry. Total RNA was isolated with TRIzol Reagent (GIBCO) according to the manufacturer's protocol. The quantity of the RNA was analyzed by absorption at 260 and 280 nm. After extraction, RNA (8–10 μg) was size fractionated by electrophoresis on 0.8% agarose gels containing formaldehyde. Prehybridization (2–4 h at 68°C) and hybridization (overnight at 68°C) of the RNA blots were performed in buffer consisting of 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 0.5% SDS. Full-length cDNA probes of OK cell type II Na-P_i cotransporter (NaPi-4) (25) and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (28) were labeled by random priming (Pharmacia) using [α-^{32}P]dCTP (DuPont-NEN). After hybridization, blots were washed twice for 5 min each time in 2× SSC + 0.1% SDS at room temperature, twice for 30 min each time in 1× SSC + 0.1% SDS at 60°C, and once in 1× SSC at room temperature for 5 min. Northern blot signals were quantitated using ImageQuant (Molecular Dynamics). Quantitated data are presented as NaPi-4 mRNA-to-GAPDH mRNA ratios to correct unequal loading.

The OK-Npt2 promoter-luciferase constructs. Genomic DNA fragments of different sizes (327 bp and 4,700 bp including 40 bp of exon 1) of the 5′-flanking region of the *OK-Npt2* (OK cell Npt2 gene = OK cell type II Na-P_i cotransporter gene) were subcloned into the pGL3 basic vector (luciferase reporter gene) from Promega as described by Hilfiker et al. (8).

Measurement of OK-Npt2 promoter activity. The *OK-Npt2* promoter activity was measured by reporter gene analysis using the above mentioned constructs. For standardization of the promoter activity the pGL3 (SV40) promoter (Promega) and the pGL3 basic vector (Promega) were used. As internal standard for transfection efficiency a β-galactosidase expressing vector pCMV-LacZ (kindly provided by Dr. S. Rusconi, University of Zurich, Switzerland) was cotransfected. Transient cell transfections, β-galactosidase reaction and the reporter gene analysis were performed as described by Hilfiker et al. (8).

Uptake measurements. Na-dependent uptake of P_i was measured on plastic dishes (35 mm; Nunc) as described previously (24).

Incubation in PTH. Parathyroid hormone (PTH fragment 1–34; Sigma) was stored as a stock solution of 10^{-4} M in a solution of 10 mM acetic acid, 0.2 mM 1,4-dithio-DL-threitol, and 1% bovine serum albumin. On the experimental day, this stock was diluted 100-fold in 10 mM acetic acid, and 20 μl of this PTH solution was added to one dish (35 mm) containing 2 ml medium. The cells were incubated in this 10^{-8} M PTH solution for 3 h. In control cells, 20 μl of vehicle (10 mM acetic acid) was used.

Incubation in dexamethasone. Dexamethasone (Sigma) was dissolved in ethanol and stored as a stock solution of 10^{-2} M. On the experimental day, this stock solution was diluted in the cell culture medium to the final solution of 10^{-6} M. Control cells received the corresponding amount of vehicle.

Incubation in DRB. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Sigma) was stored as a stock solution of 13 mM in ethanol at −20°C. On the experimental day, this stock solution was diluted 200-fold in the cell culture medium to a

final concentration of 65 μM, which was shown to effectively block RNA polymerase II (7). Control cells received the corresponding amount of vehicle.

Expression of data. All experiments were performed at least twice, and one representative experiment was chosen for publication. For the uptake experiments, Northern blots, and reporter gene analyses, statistic results are expressed as means ± SD. Significance of differences were calculated by the two-sided unpaired *t*-test ($n = 4$, unless indicated otherwise).

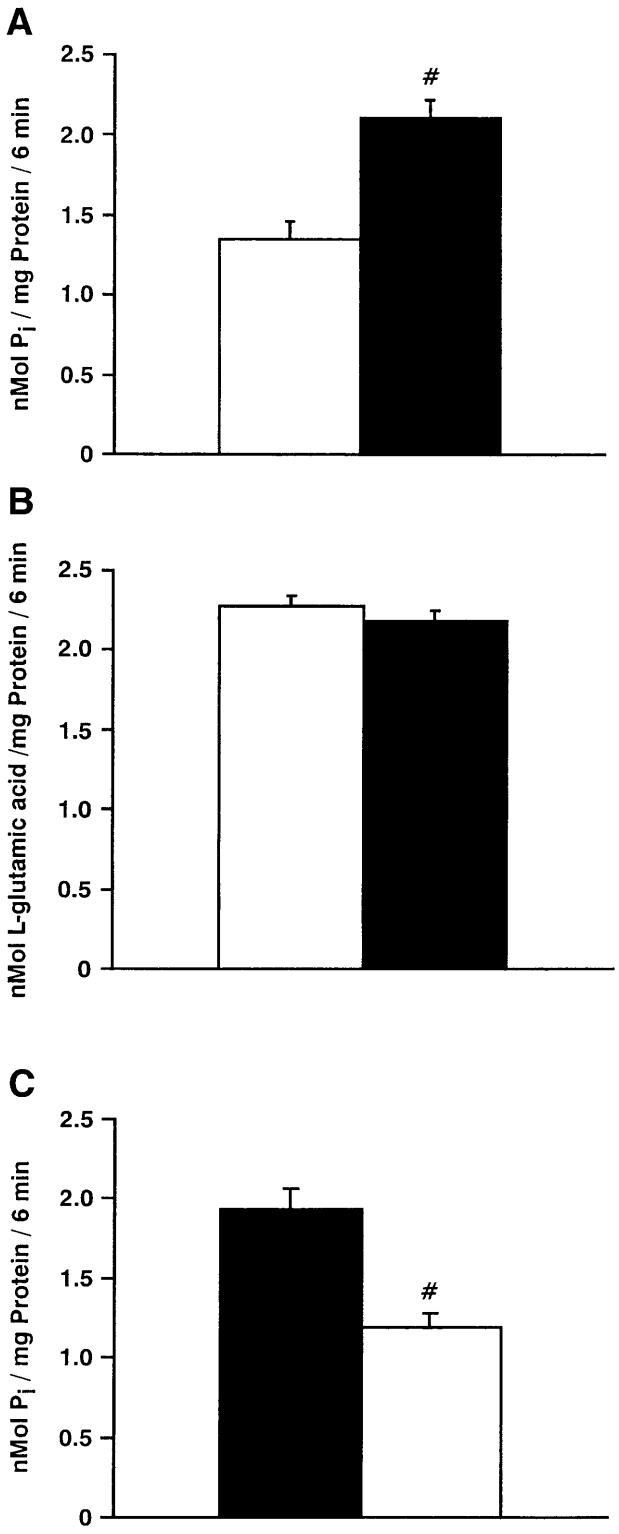

Fig. 1. Effects of isohydric changes in HCO_3/CO_2 on Na-P_i cotransport and Na-dependent L-glutamic acid cotransport in OK cells. Cells were incubated at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (open columns), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (solid columns), for 24 h. *A*: effect of HCO_3/CO_2 on Na-P_i cotransport if the cells were deprived of serum at 10% CO_2/44 mM $NaHCO_3$, pH 7.4 (for 24 h), before the experimental incubation. *B*: effect of HCO_3/CO_2 on Na-dependent L-glutamic acid cotransport using the same experimental protocol as in *A*. *C*: effect of HCO_3/CO_2 on Na-P_i cotransport when the experimental protocol was reversed in that the cells were deprived of serum at 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (for 24 h), and subsequently incubated for additional 24 h at 22 mM $NaHCO_3$/5% CO_2, pH 7.4, or they were transferred to 44 mM $NaHCO_3$/10% CO_2, pH 7.4. $^{\#}P < 0.0001$, two-sided unpaired *t*-test ($n = 4$).

RESULTS

Effect of HCO_3/CO_2 on Na-P_i cotransport. OK cells were grown to confluence and deprived of serum at 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (for 24 h). Subsequently, cells were kept at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (higher HCO_3/CO_2 condition; control), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (lower HCO_3/CO_2 condition; experimental). Figure 1*A* illustrates that incubation in lower HCO_3/CO_2 concentrations for 24 h increased Na-dependent P_i uptake significantly by about a factor of 1.5. Na-dependent transport of L-glutamic acid, which was used as a control, was similar in the control and experimental condition (Fig. 1*B*). We did also inverse the experimental protocol in that the cells were deprived of serum at 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (for 24 h), and subsequently the cells were left for additional 24 h at 22 mM $NaHCO_3$/5% CO_2, pH 7.4 or they were kept at 44 mM $NaHCO_3$/10% CO_2, pH 7.4. Using this experimental protocol, Na-P_i cotransport was also increased ~1.5-fold in cells kept at 22 mM $NaHCO_3$/5% CO_2, pH 7.4 compared with cells that were subsequently transferred to 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (Fig. 1*C*). This response was also specific in that Na-dependent transport of L-glutamic acid was similar in both groups (data not shown).

Action of PTH on Na-P_i cotransport. PTH inhibits type II Na-P_i cotransport in OK cells (19, 22). We tested whether the increased Na-P_i cotransport at the lower HCO_3/CO_2 condition is sensitive to PTH (3 h exposure; 10^{-8} M). Figure 2 shows that the percent inhibition by PTH was higher for the lower HCO_3/CO_2 condition, but the "residual" transport activity was similar in both groups. Thus a PTH-sensitive Na-P_i cotransporter was induced by the lower HCO_3/CO_2 condition.

Effect of dexamethasone on Na-P_i cotransport. Dexamethasone inhibits Na-P_i cotransport in OK cells (290). Cells were incubated at either 44 mM $NaHCO_3$/10% CO_2 or 22 mM $NaHCO_3$/5% CO_2 for 24 h in the absence or in the presence of dexamethasone (10^{-6} M). Figure 3 shows that the inhibitory effect of dexamethasone at the higher HCO_3/CO_2 condition was relatively small, but significant. On the other hand dexamethasone did not prevent the increase in Na-P_i cotransport induced by lower HCO_3/CO_2 concentrations.

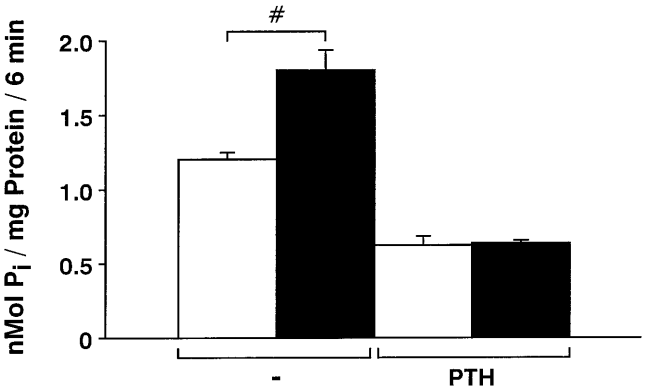

Fig. 2. Effect of parathyroid hormone (PTH, 10^{-8} M) on Na-P_i cotransport. Cells were incubated at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (open columns), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (solid columns), for 24 h. Subsequently, PTH was added for 3 h, and Na-P_i cotransport was determined. $^{\#}P < 0.0001$, two-sided unpaired *t*-test ($n = 4$).

Effect of HCO_3/CO_2 on NaPi-4 protein abundance. Western blot analyses of OK cells were performed using a polyclonal antibody. The antiserum recognized proteins with apparent molecular masses of 90–120 kDa, which are related to the NaPi-4 protein as previously demonstrated (10). Densitometric analysis of the staining intensities of NaPi-4 protein-related bands indicated a two- to threefold increase in NaPi-4 protein after incubation at lower HCO_3/CO_2 condition for 24 h compared with the control condition (Fig. 4).

Effect of HCO_3/CO_2 on NaPi-4 mRNA abundance. In the following experiments, we tested whether the increased activity of Na-P_i cotransport and the increased amount of NaPi-4 protein at lower HCO_3/CO_2 concentrations are associated with alterations in NaPi-4 mRNA abundance. NaPi-4 mRNA was analyzed by Northern blots and quantitated by densitometry and normalized to the density of the corresponding GAPDH mRNA.

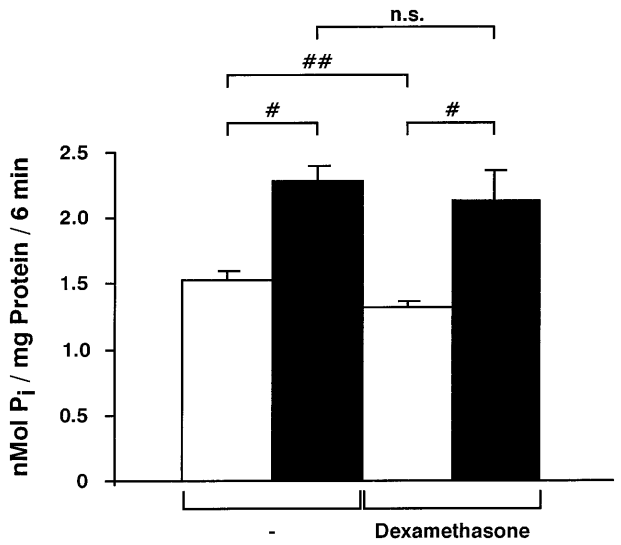

Fig. 3. Effect of dexamethasone (10^{-6} M) on Na-P_i cotransport. Cells were incubated at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (open columns), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (solid columns), for 24 h in absence (−) or presence of dexamethasone. $^{\#}P < 0.0001$ and $^{\#\#}P < 0.01$, two-sided unpaired *t*-test ($n = 4$); n.s., not significant.

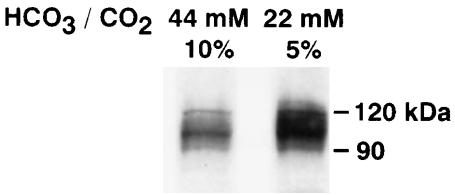

Fig. 4. Western blot analysis of NaPi-4 protein. The antiserum recognized proteins with apparent molecular masses of 90–120 kDa. Densitometric analysis of the staining intensities of NaPi-4 protein-related bands indicated a two- to threefold increase in NaPi-4 protein in cells incubated for 24 h at 22 mM $NaHCO_3$/5% CO_2, pH 7.4, compared with control cells incubated at 44 mM $NaHCO_3$/10% CO_2, pH 7.4. Similar increases of NaPi-4 protein were observed by densitometric analysis of 2 independent experiments.

Figure 5*A* shows a typical blot of six different mRNA preparations obtained after incubation for 24 h at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4, or 22 mM $NaHCO_3$/5% CO_2, pH 7.4. Figure 5*B* depicts the quantitated results of the same blot showing an approximately threefold increase in NaPi-4 mRNA abundance after incubation in the lower HCO_3/CO_2 condition for 24 h compared with the control condition.

Effects of transcriptional inhibitors. Next, we addressed the question whether the increase in NaPi-4 mRNA in cells incubated at lower HCO_3/CO_2 concentrations can be prevented by incubation in the presence of the transcriptional inhibitor DRB. Cells were incubated for 14 h at either lower HCO_3/CO_2 concentrations or higher HCO_3/CO_2 concentrations in the absence or in the presence of DRB. As shown in Fig. 6, *A* and *B*, inhibition of transcription using DRB completely prevented the approximately twofold increase in NaPi-4 mRNA observed after 14 h. Also, the increase in Na-P_i cotransport was substantially reduced in the presence of DRB (Fig. 6*C*). In control experiments, similar results were obtained using the more general transcriptional inhibitor actinomycin D (data not shown). As inhibition of transcription could completely prevent the increase in NaPi-4 mRNA abundance, the more abundant NaPi-4 mRNA at lower HCO_3/CO_2 condition seems to be the result of an increased *OK-Npt2* (OK cell type II Na-P_i cotransporter gene) transcription without concomitant changes in NaPi-4 RNA stability.

Effect of HCO_3/CO_2 on the OK-Npt2 promoter activity. To further confirm that the increased NaPi-4 mRNA abundance at lower HCO_3/CO_2 concentrations is the result of an increased *OK-Npt2* transcription, the promoter activity of *OK-Npt2* was measured. Recently the promoter of the *OK-Npt2* was cloned (8). Figure 7 shows the organization of the 5′-flanking region of the *OK-Npt2* gene. For the reporter gene analysis, two promoter fragments of different sizes (327 and 4,700 bp) including 40 bp of exon 1 were cloned in front of a luciferase reporter gene of pGL3 to estimate transcriptional activity. Both promoter fragments comprised a typical TATA box and a conserved GCAAT element, which was shown to be very important for the promoter activity (8). As depicted in Fig. 8, both promoter constructs showed a higher activity if the cells were

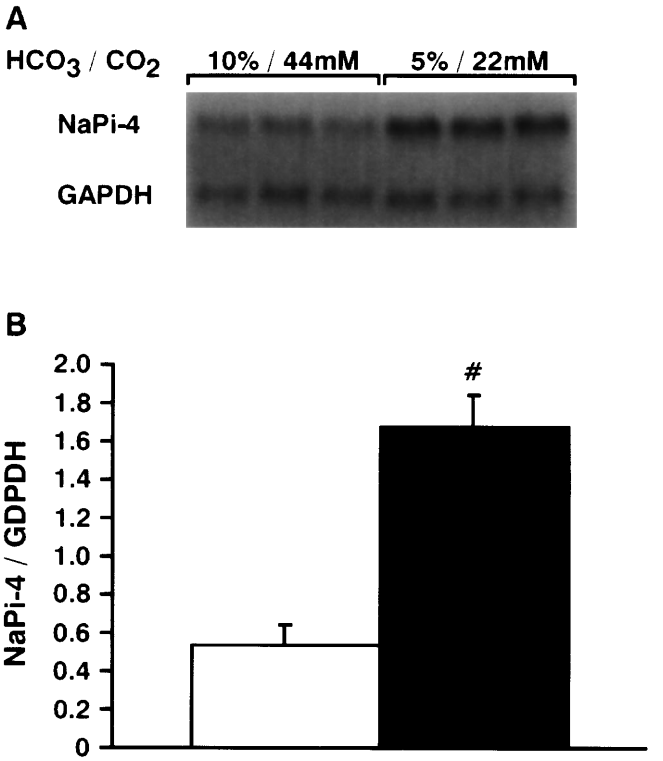

Fig. 5. Effect of isohydric changes in HCO_3/CO_2 on NaPi-4 mRNA abundance. *A*: typical blot obtained after incubation (24 h) at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (3 lanes on *left*), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (3 lanes on *right*). *B*: quantitated results of the same blot, i.e., at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (open bar), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (solid bar). mRNA levels for NaPi-4 were quantitated by densitometry and normalized to the density of the corresponding GAPDH mRNA. $^{\#}P < 0.001$, two-sided unpaired *t*-test ($n = 3$).

incubated at lower HCO_3/CO_2 concentrations compared with cells incubated at higher HCO_3/CO_2 concentrations. Thus the measurement of the *OK-Npt2* promoter activity is in complete agreement with the experiments using the transcriptional inhibitor DRB and confirms that the increased mRNA abundance at the lower HCO_3/CO_2 condition is the result of an increased *OK-Npt2* transcription.

DISCUSSION

Systemic acid-base disturbances exert important influences on the regulation of phosphate balance in large part by affecting renal phosphate excretion, i.e., renal tubular phosphate reabsorption. To gain more information about the mechanisms by which HCO_3/CO_2 might affect the renal regulation of phosphate transport, we studied the effects of isohydric changes in HCO_3/CO_2 on Na-P_i cotransport in OK cells, a well-characterized model with which to study regulation of proximal tubule Na-P_i cotransport (1, 3, 4, 19, 23). The model allows regulatory studies without the confounding influences of other systemic factors. The apically located OK cell type II Na-P_i cotransporter studied in the present experiments (NaPi-4, Ref. 25) is homologous to the major Na-P_i cotransporter molecule present in the brush-border membrane of several different species (for review, see Ref. 21).

Using this model and the experimental conditions described, we obtained the following key findings in the present studies. *1*) Incubation in the lower HCO_3/CO_2 milieu increased Na-P_i cotransport 1.5-fold. *2*) Na-dependent transport of L-glutamic acid, which was measured as a control, was similar in both conditions. *3*) The increased Na-P_i cotransport was sensitive to PTH inhibition. *4*) Incubation in the presence of dexa-

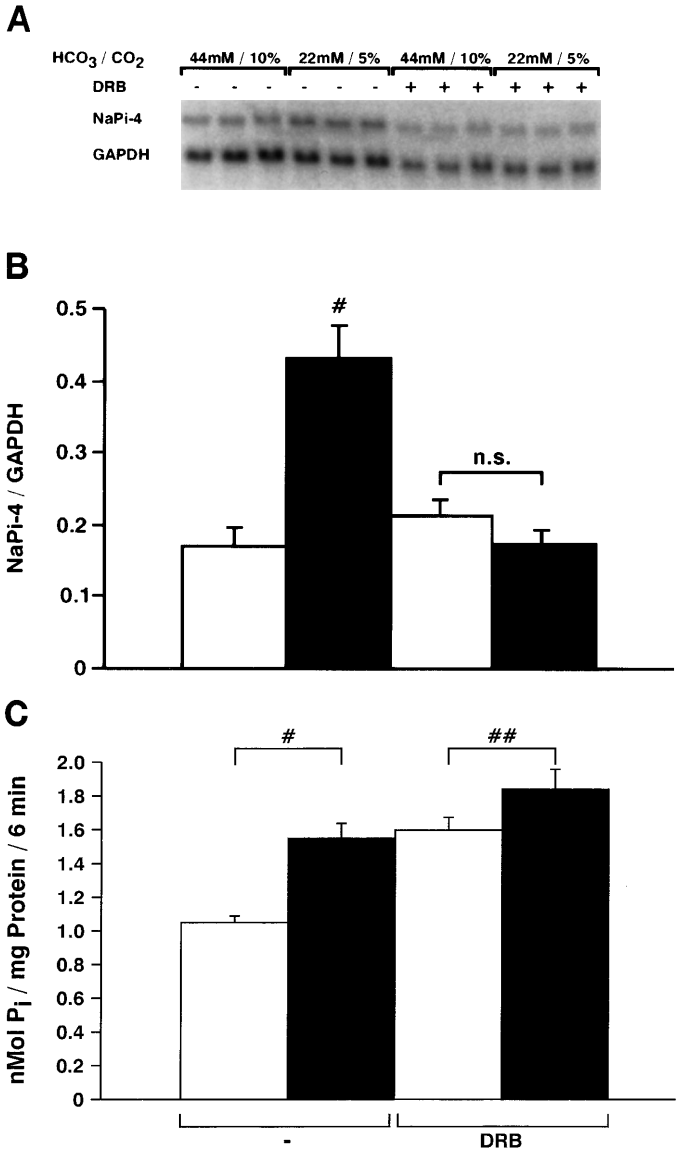

Fig. 6. Effect of the transcriptional inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) on NaPi-4 mRNA abundance and Na-P_i cotransport. Cells were incubated for 14 h at either 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (control), or 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (experimental), in absence or presence of DRB. *A*: typical Northern blot showing a triplicate for the experimental and control conditions after incubation either in absence (−) or presence (+) of DRB. *B*: quantitated results of the same blot. mRNA levels for NaPi-4 were quantitated by densitometry and normalized to the density of the corresponding GAPDH mRNA. *C*: Na-P_i cotransport for the experimental (solid bars) and control (open bars) conditions after incubation either in absence or presence of DRB. $^{\#}P < 0.001$ and $^{\#\#}P = 0.04$, two-sided unpaired *t*-test ($n = 3$).

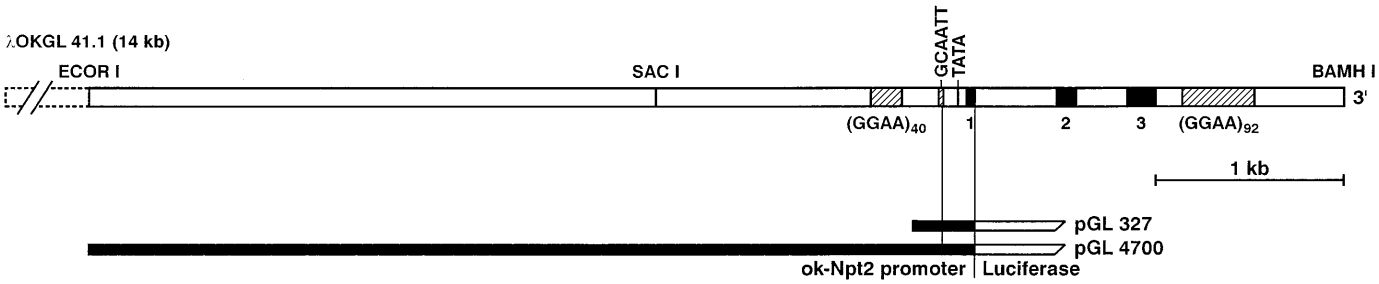

Fig. 7. Organization of the 5′-flanking region of the *OK-Npt2* gene. A genomic *Bam*H I clone of 14 kb was isolated from a OK cell λ-Dash II library. A 6,000 bp *Eco*R I-*Bam*H I subclone contains exons 1 to 3 (solid blocks *1*, *2*, and *3*) and the promoter region containing a consensus binding site for TFIID (TATA-box) at −44 and a "GCAAT" element at −139. The 5′-flanking region as well as intron 3 of the OK cell Npt2 gene exhibit long GGAA repeats of unknown function (microsatellites, hatched boxes). Two promoter fragments of different sizes (327 and 4,700 bp) both including exon 1 were cloned in front of a luciferase reporter gene of pGL3 to estimate transcriptional activity [for further details, see Hilfiker et al. (8)].

methasone did not prevent the stimulation of Na-P_i cotransport. *5*) The increased Na-P_i cotransport was paralleled by a two- to threefold increased expression of the NaPi-4 transporter protein and *6*) a two- to threefold increase in NaPi-4 mRNA abundance. *7*) The increase in NaPi-4 mRNA was prevented by incubation in the presence of DRB, which also reduced the increase in Na-P_i cotransport. *8*) The NaPi-4 gene transcription was enhanced by 50% at lower HCO_3/CO_2 concentrations.

The data demonstrate that Na-P_i cotransport in OK cells is responsive to changes in HCO_3/CO_2 without concomitant changes in pH. Regulation of Na-P_i cotransport by HCO_3/CO_2 is specific since Na-dependent transport of L-glutamic acid was not changed significantly (Fig. 1, *A*–*C*).

PTH inhibition experiments show (Fig. 2) that the stimulation of Na-P_i cotransport in cells incubated at lower HCO_3/CO_2 concentrations was entirely attributable to a PTH-inhibitable Na-P_i cotransporter, i.e., the type II Na-P_i cotransporter (21). As PTH leads to a complete disappearance of NaPi-4 protein (22), NaPi-4 cannot account for the "residual" transport activity, which cannot be inhibited by PTH (Fig. 2) and which

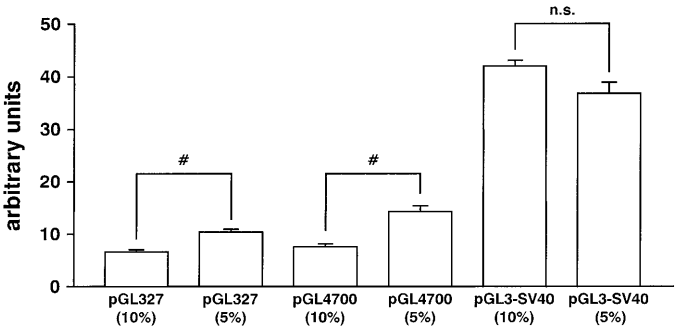

Fig. 8. Effect of isohydric changes in HCO_3/CO_2 on luciferase expression. OK cells were electroporated in presence of pGL327 and pGL4700 or in presence of the pGL-SV40 construct, which was used as a control. For each transfection, cells were equally seeded in 24-well plates containing DME-F12 and 10% FCS at 22 mM $NaHCO_3$/5% CO_2. After 2 h, cells were switched to FCS-free medium and incubated at either 22 mM $NaHCO_3$/5% CO_2, pH 7.4 (5%), or 44 mM $NaHCO_3$/10% CO_2, pH 7.4 (10%), for 24 h, and subsequently the luciferase expression was determined. $^{\#}P < 0.01$, two-sided unpaired *t*-test ($n = 3$).

accounts for ~50% of the overall transport at the higher HCO_3/CO_2 condition (Fig. 2). If the data in Fig. 1 is corrected by the residual transport activity, then the increase in NaPi-4-related Na-P_i cotransport at lower HCO_3/CO_2 concentrations was approximately twofold.

The regulation of Na-P_i cotransport by HCO_3/CO_2 can be due to changes in the number of transporter sites and/or to altered activity of expressed transporters. Western blot analysis suggests that the activation of Na-P_i cotransport by lower HCO_3/CO_2 concentrations is the consequence of an increased number of transporter molecules (Fig. 4). The two- to threefold increase in NaPi-4 protein expression is in agreement with the approximately twofold increase in NaPi-4-related Na-P_i cotransport at the lower HCO_3/CO_2 condition. As incubation at lower HCO_3/CO_2 concentrations led to a threefold increase in NaPi-4 mRNA (Fig. 5), we suggest that the increased NaPi-4 protein abundance results from an increased de novo synthesis of NaPi-4 transporter molecules. Although preliminary experiments showed that cycloheximide prevented the increase in Na-P_i cotransport in response to the lower HCO_3/CO_2 condition (data not shown), the interpretation of these data is difficult, as in Western blots performed in parallel, NaPi-4 protein was hardly detectable for either condition after exposure to cycloheximide. These data can be explained by a high turnover of the type II Na-P_i cotransporter protein also under control conditions. For this reason, effects of isohydric changes in HCO_3/CO_2 on NaPi-4 protein synthesis and on NaPi-4 protein stability and membrane delivery should be addressed in further experiments.

The threefold increase in NaPi-4 mRNA after incubation for 24 h at lower HCO_3/CO_2 concentrations (Fig. 4) suggests a regulatory step at the level of transcription or mRNA stability. As a first step toward the clarification of the mechanisms leading to the increased NaPi-4 mRNA abundance at lower HCO_3/CO_2 concentrations, we tested the effect of the transcriptional inhibitor DRB on NaPi-4 mRNA abundance and Na-P_i cotransport. As DRB prevented the approximately twofold increase in NaPi-4 mRNA observed after 14 h (Fig. 6, *A* and *B*), we conclude that the more abundant NaPi-4 mRNA at lower HCO_3/CO_2 concentrations was the result of an

increased *OK-Npt2* (OK cell type II Na-P_i cotransporter gene) transcription and that the NaPi-4 mRNA stability was not altered in parallel. As depicted in Fig. 6*C*, the relative and absolute increase in Na-P_i cotransport was reduced but not completely prevented in the presence of DRB. This suggests the participation of an additional posttranscriptional regulatory mechanism in the regulation of Na-P_i cotransport by HCO_3 and/or CO_2 (e.g., regulation at the level of translation, protein stability or membrane delivery). DRB led to an increase in transport in both conditions; we have previously observed an increase in OK cell Na-P_i cotransport activity in response to actinomycin D (10). Although we have no satisfactory explanation, the reduction of a protein synthesis-dependent step within the "normal" turnover (degradation) of the NaPi-4 protein could account for this observation.

The *OK-Npt2* promoter activity was also tested. As depicted in Fig. 8, a significant and reproducible upregulation for both promoter constructs was observed when cells were incubated at lower HCO_3/CO_2 concentrations. In agreement with the data on inhibitors, this indicates that the increased mRNA abundance at the lower HCO_3/CO_2 condition was determined to its major extent by increased activity of the *OK-Npt2* promoter.

Recently, we demonstrated that variation of hydrogen ion concentration by changing HCO_3 at constant P_{CO_2} affects Na-P_i cotransport by regulating posttranscriptional, glucocorticoid-sensitive expression of the NaPi-4 cotransporter (10). In contrast, the increased Na-P_i cotransport by lower HCO_3/CO_2 concentrations is not prevented by incubation in the presence of dexamethasone (Fig. 3), and isohydric changes in P_{CO_2} and HCO_3 exert their major regulatory influence on NaPi-4 expression and activity by affecting gene transcription. The observations on transcriptional regulation could be explained either by counterbalancing effects of hydrogen ion and HCO_3 concentrations on the transcriptional machinery of the NaPi-4 cotransporter or by direct and specific regulation of transcription by CO_2 tension. A further explanation could be that some signal transduction pathway is activated and this modifies or sets the activity of transcription factors. Additional influences on posttranscriptional regulation of NaPi-4 cotransport are affected, however, by low hydrogen and/or low HCO_3 concentration. Further studies on the regulation of promoter activity and the nature of response elements in the NaPi-4 promoter are needed to delineate which of the acid-base parameters and/or which intracellular messenger(s) is operative in affecting transcription and to determine the mechanisms by which it exerts transcriptional control.

Since cell pH could not be measured during the prolonged exposure times to different isohydric changes in HCO_3 concentrations, some uncertainties exist on whether we induced significant changes in cell hydrogen ion concentration. However, we have demonstrated in studies on rabbit proximal tubule cells that only changes in extracellular pH (by changing HCO_3 or CO_2) affect intracellular pH significantly and that isohydric changes in HCO_3/CO_2 exert no significant effect on cell pH after prolonged exposure (14). Such behavior has also been assumed in studies on monkey kidney epithelial cells (BSC-1), characterizing the bicarbonate dependence of the sodium bicarbonate symport system (11). Furthermore, direct measurements of cell pH in isolated rat hepatocytes exposed to isohydric changes in HCO_3/CO_2 documented that cell pH reaches similar values within a few minutes after changing the superfusates (6). Thus it is fair to assume that the present observation is not related to changes in cell pH.

The present results provide novel insights into the mechanisms of renal regulation of phosphate transport in chronic respiratory alkalosis, probably the most frequent acid-base disturbance in humans. Increased tubular reabsorption of phosphate is characteristic of this disorder (16), which is defined by a primary decrease in P_{CO_2} and a consecutive decrease in bicarbonate, resulting in only very small pH changes. Thus, in addition to the observed apparent renal PTH resistance (16), renal phosphate retention in chronic respiratory alkalosis might be due to CO_2/HCO_3-induced transcriptional stimulation of Na-P_i transport. Similarly, our results also provide a mechanistic explanation for the phosphaturia or decreased tubular phosphate reabsorption in respiratory acidosis (characterized by increases in P_{CO_2} and bicarbonate, see Ref. 30).

In conclusion, the cellular response to lower HCO_3/CO_2 concentrations leads to a specific stimulation of the NaPi-4 cotransport that is paralleled by an increased expression of the NaPi-4 transporter protein. In contrast to the activation of Na-P_i cotransport by acidic pH (10), this stimulation is not prevented by incubation in the presence of dexamethasone and this stimulation is characterized by an increased expression of NaPi-4 mRNA, which was shown to be exclusively the result of an increased OK cell type II Na-P_i promoter activity without concomitant changes in NaPi-4 mRNA stability.

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