Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 141-147

ADAPTATION TO PHOSPHATE DEPLETION IN OPOSSUM KIDNEY CELLS

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Received September 12, 1995

SUMMARY: Dietary phosphate restriction produces an adaptive increase in renal tubular Na/P_i cotransport. A similar adaptation occurs during phosphate depletion in opossum kidney cells, a continuous line of cultured renal epithelial cells. We investigated the cellular changes associated with adaptation to phosphate depletion in OK cells, in isolation from the complex systemic changes that occur with *in vivo* phosphate restriction. Phosphate depletion for up to 24 hours was associated with increases in Na/P_i cotransport activity, Na/P_i cotransporter mRNA, and Na/P_i cotransporter protein. Moreover, the increases in Na/P_i cotransport, and Na/P_i cotransporter mRNA and protein occurred at physiologically relevant degrees of phosphate restriction. The experimental results suggest that increases in Na/P_i cotransporter mRNA and protein may mediate the increase in Na/P_i cotransport activity in OK cells during phosphate depletion.

The kidney plays a key role in regulating P_i homeostasis. Dietary P_i restriction results in an adaptive increase in P_i reabsorption by the renal tubule [1-3]. This adaptation is achieved by an increase in Na/P_i cotransport activity in the proximal tubule apical brush-border membrane [4-6]. The adaptive increase in Na/P_i cotransport during chronic P_i restriction in rats is associated with increases in NaPi-2 mRNA and protein [7, 8]. Because *in vivo* P_i restriction is associated with multiple systemic biochemical changes (e.g., hypercalcemia, increased plasma calcitriol levels, suppressed plasma PTH levels, and increased plasma insulin levels) [3, 6, 9-12], it is difficult to

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Abbreviations: P_i, inorganic phosphate; Na/P_i cotransport, sodium-dependent P_i cotransport; OK cell, opossum kidney cell; NaPi-2, rat Na/P_i cotransporter; NaPi-4, OK cell Na/P_i cotransporter; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; SSPE, sodium chloride sodium phosphate EDTA buffer; ECL, Enhanced Chemi Luminescent; MEM, Minimum Essential Medium; EDTA, ethylenediaminetetraacetate; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; SGT, sodium-dependent glucose transporter; ELISA, enzyme-linked immunosorbent assay.

0006-291X/95 $12.00

distinguish between direct and indirect mechanisms producing an increase in Na/P_i cotransport during P_i restriction in the intact animal.

OK cells, a continuous renal tubular epithelial cell line, are a useful experimental model to investigate the cellular mechanisms involved in the adaptation to P_i restriction, in isolation from the complex systemic factors [13, 14]. The recent cloning of the OK cell Na/P_i cotransporter (NaPi-4) cDNA [15] permits quantification of NaPi-4 mRNA levels. In addition, we have raised a rabbit polyclonal antibody against NaPi-4 protein, to enable direct measurements of NaPi-4 protein levels. Using the newly available molecular tools and the NaPi-4 antibody, we investigated the cellular changes associated with the adaptive increase in Na/P_i cotransport activity during P_i depletion of OK cells.

METHODS

Cell culture. OK cells were obtained from Judith Cole (University of Missouri, Columbia, MO). The cells were grown in 35 mm culture dishes (Gibco Laboratories, Grand Island, New York) with MEM, with the addition of 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin. They were kept in an incubator at 37°C, with 95% air and 5% CO_2. The medium was changed every other day. At weekly intervals, the cells were detached from the plates with 0.05% trypsin-0.02% EDTA, and subcultured at a 1:10 dilution.

Measurement of Na/P_i cotransport. P_i transport was measured as previously reported by us [16]. After reaching confluence, the cells were rendered quiescent by a 16 h incubation with serum-free MEM containing 1 mM P_i and 1% BSA, followed by an incubation for variable periods of time in serum-free medium containing normal or low P_i concentrations. After aspirating the medium and washing, the cells were incubated at 37°C for 5 min with transport solution (137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 14.0 mM HEPES, and 0.1 mM $K_2H[^{32}P]O_4$, pH=7.4). The transport solution was aspirated, the cells washed 3 times with ice-cold 137 mM NaCl, 14 mM HEPES, pH=7.4 to stop P_i uptake, and $[^{32}P]$ uptake quantified by scintillation counting. To measure uptake of alpha-methylglucoside, 0.1 mM [^{14}C]-alpha-methylglucoside was added to the transport solution. Sodium-independent P_i or alpha-methylglucoside uptake was determined by substituting 137 mM N-methylglucamine chloride for NaCl in the transport solution. Sodium-dependent cotransport was calculated as the difference in transport with and without sodium.

Measurement of Na/P_i cotransporter mRNA levels. The full-length NaPi-2 cDNA isolated from rat kidney and cloned in pSPORT [17], was kindly provided by Heini Murer, Institute of Physiology, University of Zurich, Switzerland. The 2.5 kb insert was excised, restricted sequentially with SmaI and ApaLI to produce a 1.1 kb fragment, radiolabelled with ^{32}P-dCTP by random priming, and hybridized with OK cell RNA using Northern analysis. Total RNA was isolated from OK cells using the guanidium isothiocyanate method [18], purified by phenol and chloroform extraction, precipitated with isopropanol, and washed with 70% ethanol. Total RNA was quantified by spectrophotometry, denatured with 37% formaldehyde, separated on 1% agarose gel, and transferred to a nylon membrane. After baking the membranes at 80°C for 2 h, the blots were prehybridized with 5X SSPE, 5% SDS, and 50% formamide. After hybridization with the NaPi-2 probe, the membrane was washed X3 at room temperature with 2X SSPE, 0.1% SDS, followed by 1 wash at 42°C with 0.4X SSPE, 0.2% SDS. The blots were exposed to Kodak X-AR film, and the bands quantified by densitometry. NaPi-2 cDNA hybridized with Northern blots from OK cell RNA detected a single band at ~2.5 kb, comparable in size to NaPi-4 mRNA [15]. To provide a reference for total RNA per lane, the Northern blots were stripped and re-hybridized with a human β-actin cDNA probe.

We also measured mRNA levels for the "housekeeping gene", GAPDH. Degenerate forward (5'-AA(A/G)-TGG-GGT-GAT-GCT-GGT-GC(C/T)-G-3') and reverse (5'-CAT-GCC-AGT-GAG-(C/T)TT-CCC-GTT-C-3') primer pairs for the rat, rabbit, and human GAPDH cDNAs [19] were a kind gift of James Schafer, Department of Physiology, University of Alabama at Birmingham. After RT-PCR of OK cell RNA, these primers were used to synthesize a ~400 bp probe, which had a ~90% homology with human GAPDH cDNA. When this probe was hybridized with OK cell RNA Northern blots, it detected a single ~1.4 kb signal, representing OK cell GAPDH mRNA.

To evaluate whether P_i depletion nonspecifically induces the transcription of genes for other transporters, we also measured mRNA levels for the renal sodium-dependent glucose transporter (SGT). Forward (5'-AGC-TCA-TTC-GCA-ATG-CAG-CC-3') and reverse (5'-TGT-CCG-TGT-AAA-TCA-CCG-CC-3') primers to the published sequence of the human SGT cDNA [20](at sites of homology with the rabbit and rat SGT cDNA), were selected with the MacVector software program, and synthesized commercially (Fisher). Following RT-PCR of OK cell RNA, this primer pair was used to make a ~600 bp probe. When this probe was hybridized with OK cell RNA Northern blots, it detected a single ~2.8 kb signal, representing OK cell SGT mRNA.

Raising and purification of rabbit polyclonal anti-NaPi-4 antibody. The MacVector software program was used to obtain hydropathy plots and antigenicity plots for the putative cytosolic domain of the published amino acid sequence of NaPi-4 [15]. The 8 amino acid peptide, R-S-P-A-R-L-P-K (a.a. 566-573), was found to have both high hydrophilic and antigenic indices, making it a promising epitope for the generation of antibodies. Multi-antigen peptides (MAP) were synthesized (Research Genetics, Huntsville, Alabama), with the peptide coupled to poly-lysine to increase its immunogenicity. Four rabbits were immunized with 1 mg MAP suspended in Freund's complete adjuvant. Pre-immune sera were screened by ELISA to ensure low antibody titers. Four and six weeks after the initial immunization, the rabbits received booster MAP injections suspended in Freund's incomplete adjuvant. Blood samples obtained 10 days after the second booster revealed high antibody titers by ELISA in 3 of the 4 rabbits. The antibodies were precipitated from the antiserum with 50% ammonium sulfate saturation, and centifuged at 10,000 g for 15 min at 4°C. The precipitate was dialyzed against 10 mM Tris, pH=7.6. The dialysate was passed 3 times through an affinity column coupled to goat anti-rabbit IgG. Further purification of the antibody was performed by passing the eluate through a second affinity column to which the NaPi-4 peptide was bound. Antibody bound to the column was then eluted and dialyzed against TBS, pH=7.6. This final purified antibody was used to probe the Western blots from OK cell crude membrane protein.

Preparation of Western blots. OK cells were scraped into 5 mM HEPES/KOH, pH 7.2, and homogenized with a syringe through an 18-g needle. The resulting suspension was centrifuged (5 min at 1000 rpm). The supernatant was centrifuged for 30 min at 40,000 x g at 4°C. The pellet was resuspended in 100 mM mannitol/Tris-HCl, pH 7.2, with addition of the following protease inhibitors: 1 μM leupeptin, 1 μM soybean trypsin inhibitor, 0.1 μM pepstatin, and 1 μM aprotinin. The crude membrane preparation was frozen in liquid nitrogen until future use. It was then thawed, boiled with SDS sample buffer, run on SDS-PAGE, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat dry milk for 2 h. The membrane was then incubated with rabbit anti-NaPi-4 antibody. Finally, the membrane was incubated with goat anti-rabbit IgG (1:20,000) conjugated to horseradish peroxidase. The Western blots were developed using the ECL kit (Amersham). The purified rabbit polyclonal anti-NaPi-4 antibody detected an intense band at ~70 kDa, corresponding to the predicted size of NaPi-4 protein [15]. This band was eliminated when the antibody was preincubated with the NaPi-4 peptide (data not shown). A second band at ~84 kDa was eliminated when the crude membrane preparation was deglycosylated by an 18 h incubation at 37°C with peptide N-glycosidase F (Boehringer Mannheim)(data not shown), suggesting that it represents the glycosylated form of NaPi-4, analogous to the glycosylated form of NaPi-2 [21]. Because the P_i transport characteristics are similar for glycosylated and deglycosylated NaPi-2 protein [21], we deglycosylated the crude membrane preparation prior to loading subsequent gels, so as to quantify total NaPi-4 protein. A third (weaker) band detected at ~51 kDa probably represents a degradation product of NaPi-4 protein.

RESULTS

Time course of adaptation to Pi depletion. Na/P_i cotransport remained constant during incubation of OK cells with 1 mM P_i medium for 1 to 24 h. In contrast, incubation in P_i-free medium produced a progressive stimulation of Na/P_i cotransport activity (Figure 1A). Na/P_i cotransport in P_i-depleted cells was stimulated by 27% at 1 h, 50% at 4 h, and 121% at 24 h, as compared with the corresponding transport rates in P_i replete cells. Sodium-dependent alpha-methylglucoside cotransport did not differ between P_i-depleted and P_i-replete cells, even after a 24 h incubation (1.41±0.04 vs 1.26±0.06 nmol·mg prot $^{-1}$·5 min $^{-1}$).

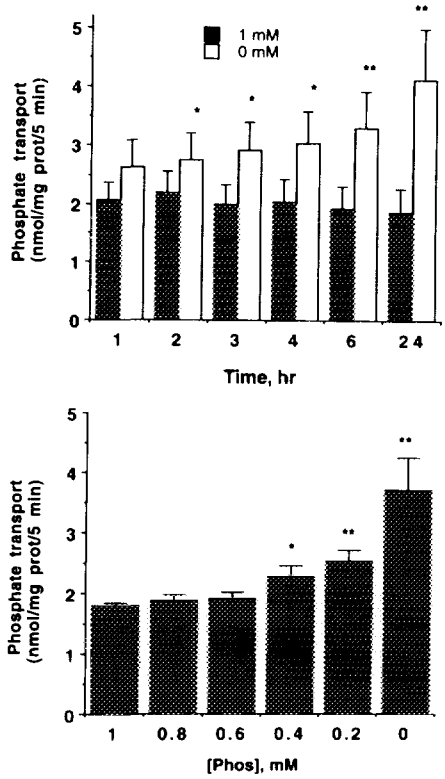

Figure 1. *Effect of Pi restriction on Na/P_i cotransport in OK cells.* OK cells were preincubated in serum-free medium containing P_i concentrations between 0 and 1 mM. The medium was aspirated, the cells were washed, and then incubated for 5 min at 37°C with transport solution, and [^{32}P] uptake quantified by scintillation counting. Upper panel. The cells were incubated with 0 or 1 mM P_i for 1 to 24 h. Lower panel. The cells were incubated for 24 h with 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM P_i. Values are means ± SE of 5 separate experiments. *P<0.05, **P<0.01 vs cells incubated with 1 mM P_i, as calculated by ANOVA.

P_i depletion also produced a time-dependent increase in NaPi-4 mRNA, that was first evident at 2 h, and increased 5-fold at 24 h, as compared with P_i-replete cells (Figure 2A). Control hybridization with human β-actin probe demonstrated that equivalent amounts of RNA were loaded from P_i-depleted and P_i-replete OK cells. P_i depletion also produced an increase in NaPi-4 protein at 4 h of P_i depletion, which increased progressively up to 24 h. In contrast, NaPi-4 protein levels remained constant in cells incubated with 1 mM P_i from 2 to 24 h (Figure 3A).

Adaptation to partial Pi restriction. The increases in Na/P_i cotransport, NaPi-4 mRNA, and NaPi-4 protein were all observed at physiologic degrees of P_i restriction. Thus, Na/P_i cotransport remained constant as extracellular P_i concentrations decreased from 1.0 to 0.6 mM, but began to increase progressively at extracellular P_i concentrations ≤ 0.4 mM (Figure 1B). Similarly, NaPi-4 mRNA levels increased progressively at extracellular P_i concentrations ≤ 0.6 mM (Figure 2B). In contrast, even total P_i depletion did not affect mRNA levels for an unrelated membrane-bound transporter, SGT, or for the "housekeeping gene", GAPDH. Finally, OK cell NaPi-4 protein levels increased progressively, as extracellular P_i concentrations were decreased to ≤0.8 mM (Figure 3B).

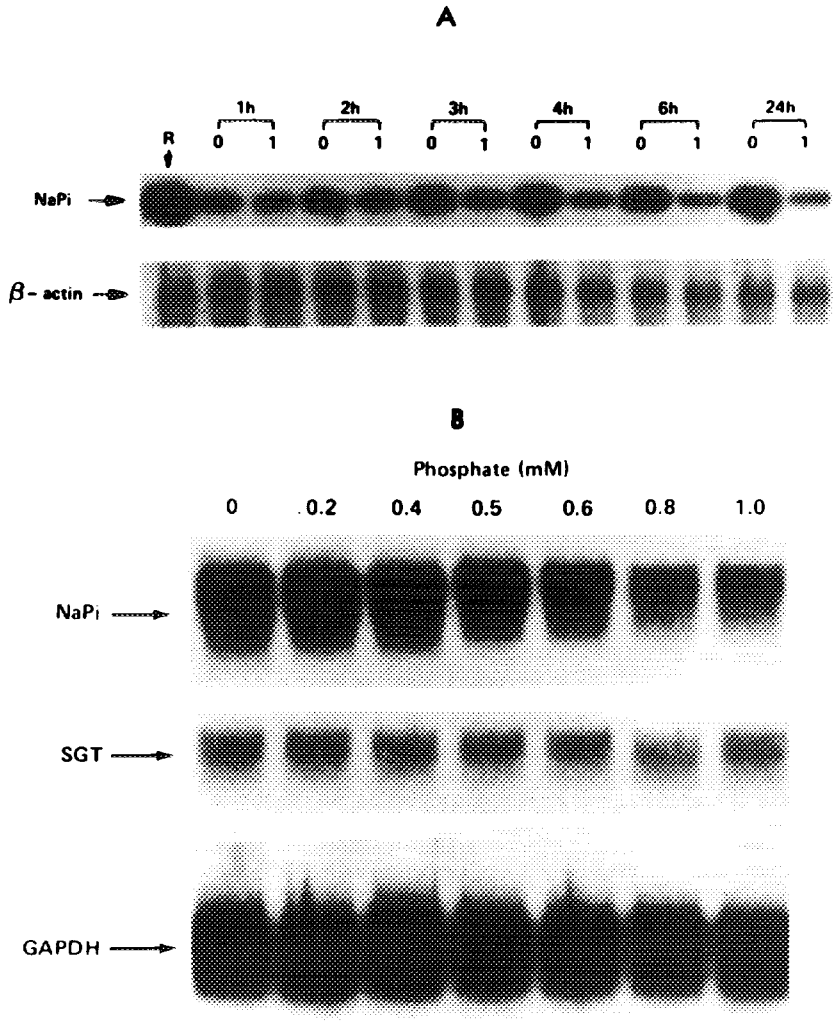

Figure 2. *Effect of Pi restriction on NaPi-4 mRNA in OK cells.* (**A**) OK cells were incubated with 0 or 1 mM P_i for 1 to 24 h. Total RNA was extracted, separated on 1% agarose (50 µg RNA/lane), and transferred to nylon membranes, and the membranes hybridized sequentially with the NaPi-2 cDNA probe and with a human β-actin probe. Lane R represents RNA from rat kidney cortex. (**B**) The cells were incubated for 24 h with 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM P_i. Total RNA was extracted, separated on 1% agarose (50 µg RNA/lane), and transferred to nylon membranes, and the membranes hybridized sequentially with the NaPi-2 cDNA probe, SGT probe, and GAPDH probe.

DISCUSSION

We have observed progressive increases in Na/P_i cotransport activity during P_i restriction of OK cells, in agreement with previous reports in OK cells [16, 22, 23]. Moreover, these adaptive increases occurred even at physiologic levels of P_i restriction, i.e., decreasing extracellular P_i from 1.0 to 0.4 mM, as shown previously by Biber, et al [22]. This process faithfully mimics the physiologic adaptation of the intact kidney, wherein an increase in tubular Na/P_i cotransport occurs with moderate degrees of hypophosphatemia [1-3]. Because the increase in Na/P_i cotransport in

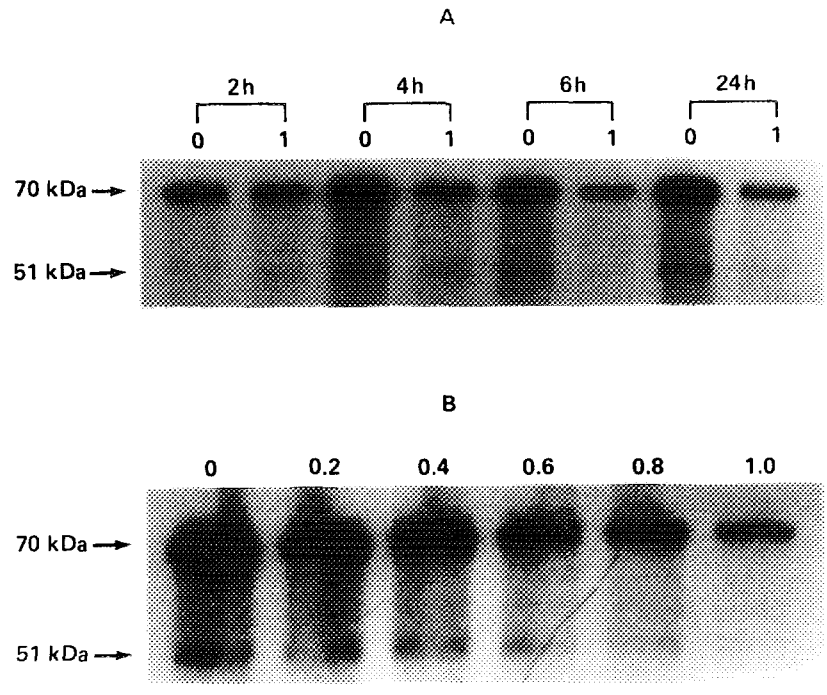

Figure 3. *Effect of Pi restriction on NaPi-4 mRNA in OK cells.* OK cells were preincubated for 24 h in serum-free medium containing P_i concentrations between 0 and 1 mM. Crude membrane protein was extracted from OK cells, solubilized in SDS buffer and boiled. The protein was run on SDS-PAGE (25 μg/lane) and transferred to nitrocellulose membrane. After nonspecific binding with 5% nonfat dry milk, the membrane was incubated with rabbit anti-NaPi-4 antibody, then with goat anti-rabbit IgG conjugated to horseradish peroxidase, and developed using the ECL kit. (**A**) The cells were incubated with 0 or 1 mM P_i for 1 to 24 h. (**B**) The cells were incubated for 24 h with 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM P_i.

P_i-depleted OK cells occurred in isolation from the systemic biochemical changes associated with *in vivo* P_i restriction, it appears to reflect an intrinsic adaptation to P_i restriction.

The increase in Na/P_i cotransport during prolonged (≥4 h) P_i depletion of OK cells was associated with parallel increases in NaPi-4 mRNA and protein, similar to the increases in NaPi-2 mRNA and protein in rats on chronic dietary P_i restriction [7, 8]. This effect of P_i depletion appeared to be specific for Na/P_i cotransport, since sodium-dependent alpha-methylglycoside uptake, as well as SGT mRNA levels, were unchanged. Moreover, the increases in NaPi-4 mRNA and protein in OK cells occurred at physiologically relevant degrees of P_i depletion. These observations suggest that the adaptive increase in Na/P_i cotransport in P_i restriction in OK cells at ≥4 h involves increased production of the specific Na/P_i cotransporter mRNA and protein. In contrast, the early (up to 3 h) increase in Na/P_i cotransport activity precedes the increase in NaPi-4 protein; it may be modulated by translocation of NaPi-4 from intracellular membranes to the plasma membrane or by post-translational modification of the transporter (e.g., phosphorylation). Further studies will be required to characterize more precisely the cellular mechanisms mediating the intrinsic adaptations to P_i restriction in OK cells.

ACKNOWLEDGMENTS

The studies reported in this manuscript were supported in part by a research grant from Dialysis Clinic, Inc., Nashville, Tennessee. The authors thank David G. Warnock, M.D. for his ongoing advice, support, and encouragement; James L. Lewis, M.D. for assistance in the design of PCR primers; and Angela Myracle and Joyce Watkins for technical support.

REFERENCES

1. Steele, T. H., and DeLuca H.F. (1976) J. Clin. Invest. 57, 867-874.
2. Troehler, U., Bonjour J. P., and Fleisch H. (1976) J. Clin. Invest. 57, 264-273.
3. Allon, M., Rodriguez M., and Llach F. (1990) Kidney Int 37, 14-20.
4. Levine, B. S., Ho K., Hodsman A., Kurokawa K., and Coburn J.W. (1984) Min. Electrolyte Metab. 10, 222-227.
5. Caverzasio, J., and Bonjour J. P. (1985) Pflug. Arch. 404, 227-231.
6. Allon, M., and Hruska K. A. (1991) Diabetes 40, 1134-1140.
7. Werner, A., Kempson S. A., Biber J., and Murer H. (1994) J. Biol. Chem. 269, 6637-6639.
8. Levi, M., Lotscher M., Sorribas V., Custer M., Arar M., Kaissling B., Murer H., and Biber J. (1994) Am. J. Physiol. 267, F900-F908.
9. Allon, M. (1992) J. Am. Soc. Nephrol. 2, 1593-1600.
10. Rader, J. I., Baylink D. J., Hughes M. R., Safilian E. F., and Haussler M. R. (1979) Am. J. Physiol. 236, E118-E122.
11. Kurnik, B. R. C., and Hruska K. A. (1984) Am. J. Physiol. 247, F177-F182.
12. Bushinsky, D. A., Nalbantian-Brandt C., and Favus M. J. (1989) Am. J. Physiol. 256, F285-F289.
13. Murer, H., Werner A., Reshkin S., Wuarin F., and Biber J. (1991) Am. J. Physiol. 260, C885-C899.
14. Murer, H. (1992) J. Am. Soc. Nephrol. 2, 1649-1665.
15. Sorribas, V., Markovich D., Hayes G., Stange G., Forgo J., Biber J., and Murer H. (1994) J. Biol. Chem. 269, 6615-6621.
16. Allon, M., and Parris M. (1993) Am. J. Physiol. 264, F404-F410.
17. Magagnin, S., Werner A., Markovich D., Sorribas V., Stange G., Biber J., and Murer H. (1993) Proc. Natl. Acad. Sci. 90, 5979-5983.
18. Chomcyznski, P., and Sacchi N. (1987) Anal. Biochem. 162, 156-159.
19. Tso, J. Y., Sun X. H., Kao T. H., Reece K. S., and Wu R. (1985) Nucleic Acids Res. 13, 2485-2502.
20. Hediger, M. A., Coady M. J., Ikeda T. S., and Wright E. M. (1987) Nature 330, 379-81.
21. Hayes, G., Busch A., Lotscher M., Waldegger S., Lang F., Verrey F., Biber J., and Murer H. (1994) J. Biol. Chem. 269, 24143-24149.
22. Biber, J., Forgo J. and Murer H. (1988) Am. J. Physiol. 255, C155-C161.
23. Quamme, G., Biber J., and Murer H. (1989) Am. J. Physiol. 257, F967-F973.