Basolateral Phosphate Transport in Renal Proximal-Tubule-Like OK Cells[1]

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It is generally assumed that phosphate (Pi) effluxes from proximal tubule cells by passive diffusion across the basolateral (BL) membrane. We explored the mechanism of BL Pi efflux in proximal tubule-like OK cells grown on permeable filters and then loaded with ^{32}P. BL efflux of ^{32}P was significantly stimulated ($P < 0.05$) by exposing the BL side of the monolayer to 12.5 m*M* Pi, to 10 m*M* citrate, or by acid-loading the cells, and was inhibited by exposure to 0.05 m*M* Pi or 25 m*M* HCO_3; by contrast, BL exposure to high (8.4) pH, 40 m*M* K^+, 140 m*M* Na gluconate (replacing NaCl), 10 m*M* lactate, 10 m*M* succinate, or 10 m*M* glutamate did not affect BL ^{32}P efflux. These data are consistent with BL Pi efflux from proximal tubule-like cells occurring, in part, via an electro-neutral sodium-sensitive anion transporter capable of exchanging two moles of intracellular acidic H_2PO4^- for each mole of extracellular basic $HPO4^=$ or for citrate.

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Data from experiments in renal cell monolayers in culture and from renal cortical membrane vesicles indicate the existence of both Na^+-independent and Na^+-dependent phosphate (Pi) transport systems at the basolateral (BL) membrane of proximal tubule cells (1–5). We have recently shown that in monolayers of OK cells, the apical Na-dependent Pi transporters are upregulated in response to Pi depletion, but neither the effective basal-lateral Pi permeability nor transepithelial Pi transport are altered (6). A basal-lateral Na^+-independent Pi transport system was reported to be inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS), suggesting the presence of Pi-anion exchange (3, 4, 7). The counter anion for this exchanger has not been yet identified. The Na^+-dependent system most probably mediates entry of Pi from the extracellular fluid into the cells, rather than Pi efflux from the cells (5, 8).

Although it is widely accepted that the apical NaPi co-transport system is the rate-limiting step for epithelial Pi transport (9), the aim of this study was to explore the mechanism by which Pi exits across the BL membrane of proximal tubule-like OK cells.

Methods

Cell Cultures. OK cells were grown in monolayers on 75-cm^3 plastic Falcon flasks in 6 ml of Dulbecco's modified Eagle's medium (DMEM):Ham F-12 (1:1) containing 1 m*M* Pi, 22 m*M* $NaHCO_3$, 20 m*M* Hepes, 2 m*M* L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS). To prepare subsequent cultures, the confluent monolayers grown on 75-cm^3 flasks were trypsinized (0.25% trypsin and 1 m*M* EDTA.4Na) for 3–5 min, washed three times by repeated centrifugations in the above described growing medium, and seeded on Cyclopore^R Falcon inserts at a density of 5×10^5 cells/well.

The top (apical) compartment of permeant polyester membrane filter inserts (25 mm diameter, 0.45 μm pore diameter) was filled with 2 ml of DMEM:F-12. Each insert was placed in a plastic plate well containing 2 ml of growing medium. This system provides access to both sides of the cell monolayer growing on the top surface of the insert membrane. The cells were kept in an incubator gassed with 5% CO_2 in air at 37°C . The medium was changed 72 hr after seeding and every 24 hr thereafter. Cell monolayers reached confluence over a period of 7–10 days as verified under the microscope. As shown previously, in these monolayers, apical uptake of Pi is upregulated in response to Pi depletion, there is net apical-to-BL Pi transport, but net BL-to-apical mannitol flux, and asymmetry in BL (33% ± 5% in 60 min) and apical (10% ± 5% in 60 min) efflux of ^{32}Pi from the cells (6).

Assay of ^{32}P Efflux. The confluent monolayers were incubated overnight (14–16 hr) in FBS-free Ham F12 medium. Monolayers on inserts were then exposed at the apical

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side to 2 ml of Hepes-buffered saline (HBS) containing (in millimoles/liter): 140 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgCl_2$, 15 Hepes, and 0.1 ^{32}Pi (2–4 μci/ml), pH 7.4, for 1 hr to load the cells with ^{32}P. The side facing the well (BL side) remained in Ham F12 medium with 1 m*M* Pi. After loading with ^{32}P, the media were aspirated from both sides of the inserts, and the inserts were rinsed three times with 100 m*M* ice-cold Mg gluconate. The inserts were then placed at 37°C in the wells of a clean 6-well culture plate containing 2 ml/well of either control or experimental HBS-like solution; 2 ml of the 1 m*M* Pi, Ham F12 medium were placed on the apical side. A 1-ml sample of the solution in the BL compartment was collected 5, 10, 30, and 60 min after the last Mg gluconate wash. The remaining solution was aspirated and replaced by 2 ml of fresh medium. The collected sample was mixed with 10 ml of scintillation fluid (Aquasol) for counting of radioactivity. At 60 min, and in some experiments at other sampling times, the apical solution was collected, the cell monolayer was lysed in 2 ml of 0.2 *N* KOH containing 0.25% Triton X-100, and was then mixed by shaking for at least 30 min. The radioactivity in the monolayers was measured by counting 1.5 ml of the KOH-Triton extract in 10 ml of scintillation fluid. The remaining extract was used to measure the cell protein concentration using the Coomassie blue procedure (Bio-Rad, Hercules, CA) and the Pi specific activity. The fractional BL efflux of cellular ^{32}P was calculated as the ratio of the ^{32}P collected at each time point in the BL solution to the total ^{32}P present measured as the sum of that collected in BL and apical solutions and that recovered in the cell monolayer. Transepithelial fluxes of ^{32}P were minimized by the frequent changes in BL solution and by the relatively large pools (usually 2 μ*M*oles) of unlabeled Pi present in apical and BL compartments. In some experiments, to measure the cell Pi specific activity and calculate absolute rates of Pi efflux, the cell proteins were precipitated with 6% perchloric acid (PCA), and the organic ^{32}P present in the PCA soluble fraction was adsorbed on charcoal (Norit A in 1 *N* HCl, 1:1, v:v). The concentration of Pi in this adsorbed extract was measured using a phosphomolybdate assay (10); the ^{32}P concentration in another aliquot of the same extract was determined by liquid scintillation counting.

BL Pi efflux was measured under the following experimental conditions: when the concentration of Pi in the HBS solution bathing the BL side of the monolayer was altered from 1 to 0.05 or to 12.5 m*M* Na_2HPO_4 (reducing NaCl to 127.5 m*M*); to investigate the electrical potential dependence of Pi efflux, cells were depolarized by exposing their BL side to a HBS-like solution (containing 1 m*M* Pi) in which the KCl was increased to 40 m*M* while the NaCl was reduced to 100 m*M*; when 10 of the 140 m*M* NaCl in a HBS solution bathing the BL side was replaced by 10 m*M* of either sodium lactate, glutamate, succinate, or citrate (Pi remaining at 1 m*M*); when all the Cl^- in a HBS solution containing 1 m*M* Pi was replaced by gluconate, an impermeant anion; the sensitivity of Pi efflux to changes in pH of the solution bathing the BL side of the cells was tested by comparing efflux into HBS solutions containing 1 m*M* Pi at pH 8.4 and 7.4, respectively; when the concentration of $NaHCO_3$ at the BL side was increased from 0 to 25 m*M* (while the NaCl was decreased to 115 m*M* in the nominal absence of CO_2); and finally, the effect of intracellular acidification on BL Pi efflux was studied in cell monolayers exposed for 1 hr to an apical HBS solution with 0.1 m*M* ^{32}Pi and 10 m*M* NH_4Cl and was exposed thereafter, on both apical and BL sides, to a 15-m*M* Hepes-buffered (pH 7.4) Na^+-free solution containing 140 m*M* choline Cl and 1 m*M* potassium Pi.

Materials. The OK cell line was kindly provided by Drs. J. Biber and H. Murer (Zurich, Switzerland). Cell culture supplies were purchased from Falcon (BD Bioscience, Bedford, MA) or Gibco (Grand Island, NY). Radio-labeled materials were obtained from New England Nuclear (Boston, MA). Organic and inorganic chemicals used for transport assays were purchased from either Sigma (St. Louis, MO) or Baker (Sanford, ME).

Statistics. Data were analyzed using paired or unpaired *t* test. A value of $P < 0.05$ was considered statistically significant. All values are presented as means ± SE.

Results

Exposure of the BL side of the monolayers to a solution containing a high concentration of Pi accelerated absolute as well as fractional efflux of ^{32}P (Fig. 1, A and B). Efflux was a multiexponential function of washout time. A rapid phase (0–5 min) could be distinguished from a slower phase (10–60 min). At 5 min, 19.2% ± 2.3% of the cell ^{32}P (14.6×10^4 dpm/mg protein) had exited through the BL membranes of cells exposed to 12.5 m*M* Pi in the BL solution compared with 15% ± 2.6% in cells exposed to 1 m*M* Pi solution ($P < 0.005$). At 60 min of incubation, 48.6% ± 4.4% of the cellular ^{32}P had exited through the BL side of cells exposed to 12.5 m*M* Pi compared with 33.5% ± 3.0% (10×10^4 dpm/mg) in cells exposed to a solution containing 1 m*M* Pi ($P < 0.005$; Fig. 1A). Exposure of the BL side of the monolayers to a solution containing 0.05 m*M* instead of 1 m*M* Pi reduced ^{32}P efflux to 2.7% ± 0.05% at 5 min and to 7% ± 1% at 60 min ($P < 0.01$; Fig 1A). Absolute rates of Pi efflux (Fig. 1B) were calculated from the sums of the ^{32}Pi recovered from the BL solution divided by the cell Pi-specific activity measured in the PCA-soluble charcoal-adsorbed extracts of the cell monolayers. Kinetic analysis of the transstimulatory effect of BL Pi on the Pi efflux rates (Fig. 1C) were based on the data obtained after 5 min of washout and were: V_{max} = 6.64 ± 0.03 nmol/mg at 5 min; K_m 0.323 ± 0.003 m*M* BL Pi.

Exposure of the BL side of the cells to solutions containing 40 m*M* KCl had no effect on ^{32}P efflux (Table I). Relative rates of BL ^{32}P efflux were 15.0% ± 2.6% vs 15.3% ± 2.5% ($P > 0.8$) at 5 min and 33.5% ± 3% vs 35.5% ± 2.7% ($P > 0.4$) at 60 min, for control and experimental conditions, respectively.

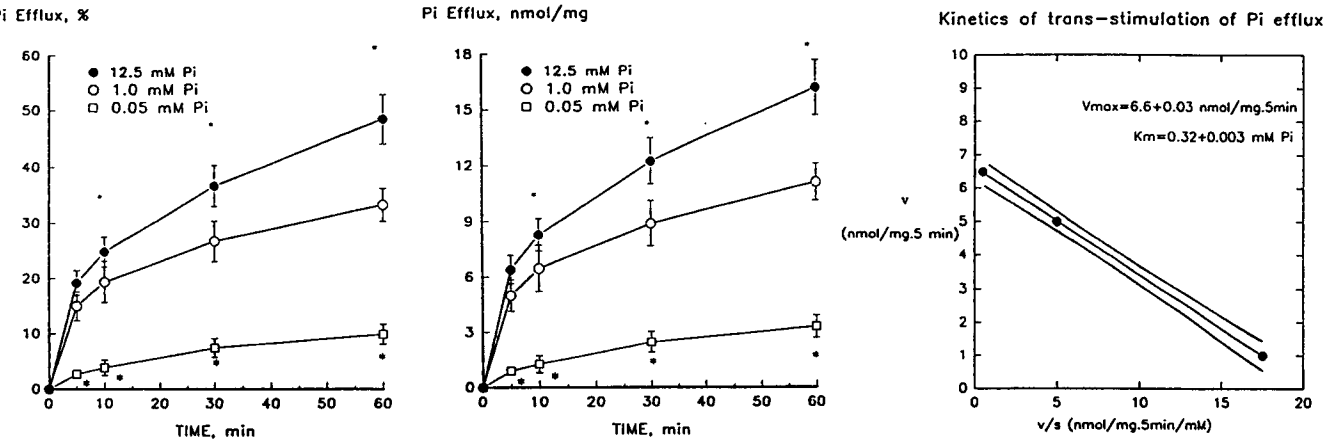

Figure 1. Trans-stimulation of BL Pi efflux by phosphate. Monolayers were incubated for 1 hr in uptake medium containing 0.1 m*M* ^{32}Pi to load the cells with ^{32}Pi. The time course of ^{32}P efflux into a BL solution containing either 0.05, 1, or 12.5 m*M* Pi was measured. (A) Each point is the mean ± SE of the cumulated fraction of intracellular ^{32}P (at time 0) effluxing into the solution bathing the BL side of the cells, for three separate experiments, performed in duplicate. (B) Absolute rates of Pi efflux were calculated using the data in A, the cell Pi uptake, and the specific activity of cell Pi, measured as described in 'METHODS." (C) Kinetics of trans-stimulation of Pi efflux across the BL membrane by the Pi concentration in the solution to which it was exposed. Data were derived from those presented in B. $^{*}P < 0.025$; $^{**}P < 0.05$.

Table I. Percentage of the Intracellular ^{32}P That Effluxed Across the Basolateral Cell Membrane

	At 5 min	At 60 min
Control	10.5 ± 0.5	29.6 ± 3.4
Gluconate	12.5 ± 2.2	32.3 ± 4.6
Control	15.0 ± 2.6	33.5 ± 3.0
40 mM KCl	15.3 ± 2.5	35.5 ± 2.7
Control	11.6 ± 2.5	28.5 ± 0.9
Glutamate	8.7 ± 1.6	30.0 ± 2.1
Lactate	10.3 ± 1.5	28.6 ± 1.4
Control	11.4 ± 1.0	38.1 ± 4.2
pH 8.4	12.3 ± 1.6	40.0 ± 4.4
Control	12.2 ± 2.2	41.2 ± 3.4
Succinate	13.7 ± 2.6	42.9 ± 2.4

Note. For all comparisons versus paired controls, $P > 0.1$.

BL efflux of ^{32}P into an HBS solution containing physiological concentration of bicarbonate (25 m*M* replacing NaCl in the nominal absence of CO_2) was 8.5% ± 1.1% at 5 min and 17.1% ± 1.2% (5×10^4 dpm/mg) at 60 min compared with 14.2% ± 1.7% efflux at 5 min and 21.7% ± 3.0% at 60 min (6.5×10^4 dpm/mg, $P < 0.01$) into a bicarbonate-free solution (Fig. 2) of the same pH (140 m*M* NaCl and 15 m*M* Hepes-Tris, pH 7.4; $P < 0.05$).

When exposed to a BL solution with an alkaline pH (8.4), 12.3% ± 1.6% ($P > 0.05$) of the ^{32}P in the cells (32×10^4 dpm/mg) effluxed in 5 min; at a neutral pH (7.4), ^{32}P efflux was 11.4% ± 1.04%. At 60 min, the corresponding values were 40.0% ± 4.4% and 38.1% ± 4.2% ($P > 0.1$; Table I).

To evaluate the influence of intracellular pH changes, we measured BL efflux of ^{32}P from acid-loaded (10 m*M* NH_4Cl) and control OK cells into a solution containing 140 m*M* choline chloride, 1 m*M* Pi, and 15 m*M* Hepes, pH 7.4 (Fig. 3). An identical solution bathed the apical cell surface. Cell ^{32}P uptake ($30 \pm 2 \times 10^4$ dpm/mg) was not significantly altered by ammonium loading ($29 \pm 2 \times 10^4$ dpm/mg, $P >$

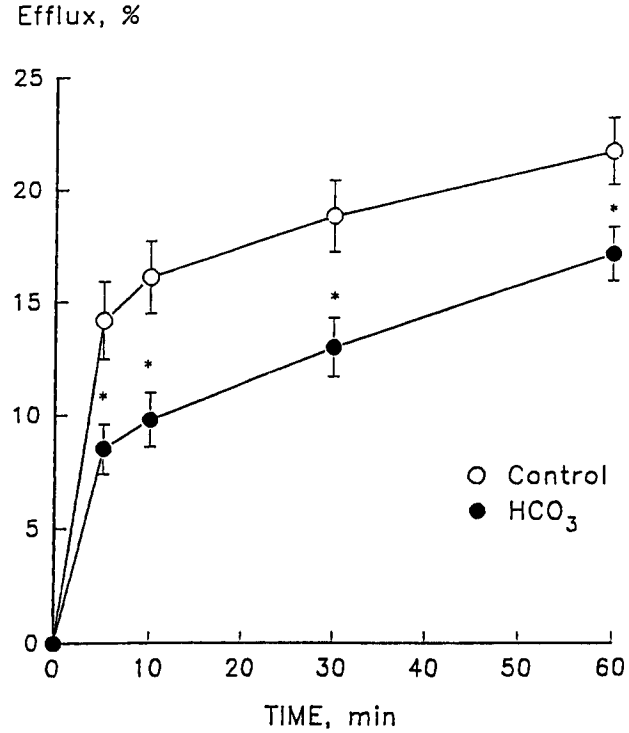

Figure 2. Inhibition of BL Pi efflux by HCO_3^-. ^{32}Pi efflux into a solution containing 1 m*M* Pi, 25 m*M* HCO_3^-, 15 m*M* Hepes, and 115 m*M* NaCl, pH 7.4, and into a HCO_3-free solution containing 1 m*M* Pi, 15 m*M* Hepes, and 140 m*M* NaCl, pH 7.4, $^{*}P < 0.025$. Data are for three experiments performed in duplicate. Note that a 15 m*M* Hepes, 140 m*M* NaCl, HCO_3-free, alkaline solution (pH 8.4) had no significant effect on BL ^{32}Pi efflux (Table I).

0.8). In the presence of 140 m*M* choline chloride, BL efflux of ^{32}Pi was 5% ± 1.1% at 5 min and 12% ± 1.2% at 60 min, lower ($P < 0.05$) than observed into 140 m*M* NaCl-containing HBS solutions (Table I, Controls) where it was 10.5%–15% at 5 min and 28.5%–38.1% at 60 min. In addition, BL efflux of ^{32}P was significantly higher when occurring from acid-loaded than from control cells (8.1% ±

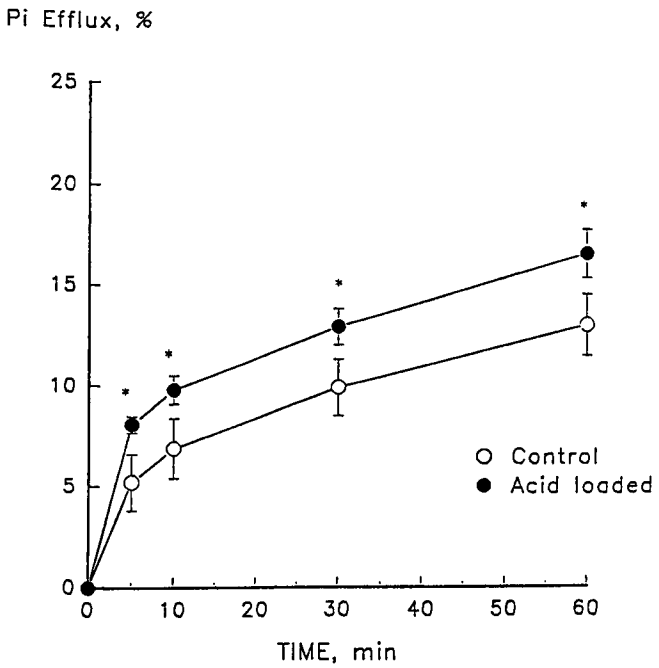

Figure 3. Stimulation of BL Pi efflux by intracellular acidification. To load the cells with ^{32}P and NH_4Cl, the apical side was exposed for 60 min to the HBS solution described in "METHODS" in which 10 m*M* NaCl were replaced by 10 m*M* NH_4Cl. The values represent BL ^{32}Pi efflux from NH_4Cl-loaded and control cell monolayers exposed on both sides to a solution containing 140 m*M* choline chloride, 1 m*M* Pi, and 15 m*M* Hepes, pH 7.4, $*P < 0.025$. Data are from three separate experiments performed in duplicate.

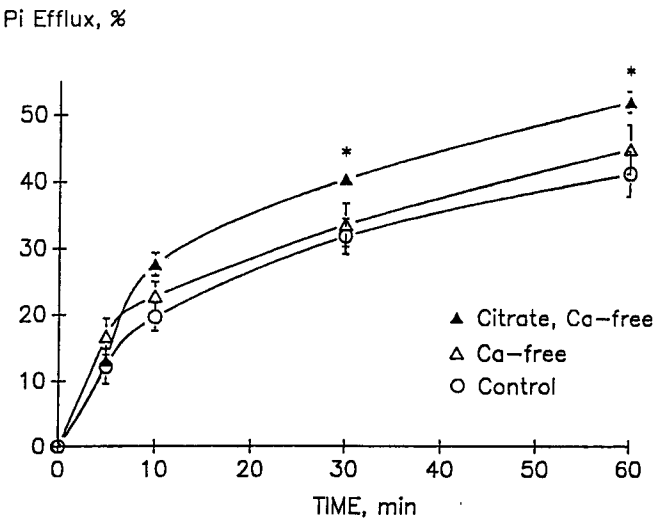

Figure 4. Effects of Ca^{2+} and citrate on BL Pi efflux. The time course of ^{32}P efflux was measured in a control solution containing 140 m*M* NaCl, 2.8 m*M* Ca^{2+}, and in two experimental solutions: one containing 120 m*M* NaCl, 10 m*M* citrate, and 0 Ca^{2+}, the other containing 140 m*M* NaCl, 0 citrate, 0 Ca^{2+}, and 1 m*M* EGTA. Measurements were performed over 60 min. Values are the means ± SE of fractional ^{32}Pi efflux from three separate experiments performed in duplicate. $*P < 0.05$.

0.4% vs 5.2% ± 1.4% at 5 min, 16.4% ± 1.2% vs 12.9% ± 1.5% at 60 min, $P < 0.05$).

Replacement of Cl^-, the major extracellular anion, by gluconate, an impermeant anion, or by permeant anions such as glutamate, succinate, and lactate in the solution bathing the BL side did not affect ^{32}P efflux (Table I). Only citrate accelerated the absolute as well as the fractional efflux of ^{32}P (Fig. 4). Because citrate is a calcium chelator, we tested the effect of citrate on ^{32}P efflux in the absence of extracellular Ca^{2+}(Ca-free HBS containing 1 m*M* EGTA). The cell ^{32}P (35×10^4 dpm/mg) effluxed into a citrate-containing Ca^{2+}-free HBS solution at a significantly higher rate than into a Ca^{2+}-free, 1 m*M* EGTA HBS solution without citrate (52.1% ± 1.6% vs 41.2% ± 3.4% at 60 min, $P < 0.05$; Fig. 4). The absence of extracellular Ca^{2+} (and presence of 1 m*M* EGTA) had no significant effect on ^{32}P efflux ($P > 0.5$; Fig. 4). These results indicate that ^{32}P efflux across the BL membrane of OK cells is stimulated specifically by citrate but not by succinate nor by a low $[Ca^{2+}]_o$.

The efflux of Pi from the cells to the apical compartment after 60 min was 10% ± 5% of initial cell Pi and did not significantly vary in the various experimental conditions.

Discussion

Our data show (Fig. 1) that phosphate in the solution bathing the BL membrane trans-stimulates Pi efflux across this cell membrane, consistent with the existence of a carrier-like Pi transport system capable of Pi-Pi exchange at this membrane. Similar changes in BL Pi efflux were observed at 5 and 60 min of incubation, indicating that the influence of transepithelial ^{32}P fluxes on these data was minimal.

The trans-stimulation had a K_m of 0.32 m*M*. Thus, 1 m*M* BL Pi exerts a near maximal trans-stimulatory effect (Fig. 1, A and B). The V_{max} was 6.6 n*M*oles/mg at 5 min, severalfold higher than the V_{max} reported for apical sodium-Pi co-transport in OK cells (3). Schwab *et al.* (4) demonstrated trans-stimulation of ^{32}P uptake in rat BL membrane vesicles preloaded with 2 m*M* Pi. A carrier, exchanging sulfate for $HPO4^=$, was found in BL membrane vesicles of rat kidney cortex (7). Experiments performed on isolated proximal tubule cells obtained from chicks revealed that the phosphate analog vanadate inhibits Pi efflux (11), further supporting the presence of a specific carrier protein for Pi exit from the cells.

The fact that changes in the BL K^+ concentration did not alter Pi efflux in OK cells suggests that the exchanger is not affected by changes in cell membrane potential and that it is electrically neutral. This observation is concordant with that of Butterworth *et al.* (11) who reported that addition of the K^+ ionophore valinomycin to isolated proximal tubule chick cells suspended in K^+-free medium (to increase electronegativity within the cell) had no effect on Pi efflux. However, Na^+-independent, electrogenic Pi transport has been reported to occur in canine renal cortical BL membrane vesicles (4).

The addition of HCO_3^- to the media bathing the BL membrane (in the nominal absence of CO_2) decreased Pi efflux in the absence of extracellular alkalinization. An in-

hibitory effect of HCO_3^- on Pi transport has been shown to occur in intact proximal tubules (12) and in renal cortical BL membrane vesicles (13). It may be due to a reduction in cellular acid phosphate $[H_2PO_4^-]_i$ when the cell alkalinizes due to HCO_3^- entry, as has previously been observed in proximal tubule and other epithelial cells where basal-lateral HCO_3 transport has a dominant influence on cell pH (14, 15). By contrast, acidification of the cell interior induced by NH_3 exit from NH_4^+-loaded cells, the major process determining OK cell pH changes when in the absence of extracellular sodium (15), increased Pi efflux, probably by raising the intracellular acid phosphate $[H_2PO_4^-]_i$ concentration. However, changing the pH of the BL solution from 7.4 to 8.4, which produces only a small (22%) increase in the extracellular basic phosphate $[HPO_4^=]$ concentration (to 0.8 m*M*), did not significantly affect ^{32}P efflux, as expected from the low K_m of the exchanger for extracellular Pi (0.32 m*M*). These observations are consistent with an exchange of extracellular basic phosphate for intracellular acid phosphate. It should be noted that an electroneutral exchange of 2 moles of $H_2PO_4^-$ for 1 mole of $HPO_4^=$ results in a net extrusion of phosphate and of acid from the cell, and therefore may contribute not only to net BL efflux of Pi, but also to the regulation of cell (pH), similar to the cell alkalinizing effect of predominant apical influx of basic $HPO_4^=$ via NaPi co-transport (16).

The presence of a Pi exchanger in the BL membrane of the proximal tubule (17) capable of exchanging 2 moles of intracellular H_2PO_4- for 1 mole of extracellular $HPO_4^=$ may explain why Pi does not leak rapidly out of the cells, and the $[Pi]_i$ remains stable for several hours, when the kidney is perfused with a Pi-free solution (18, 19).

The finding that BL ^{32}P efflux is slower into choline than into sodium-containing solutions suggests that this BL Pi exchanger may be modulated by extracellular sodium, similar to other anion exchangers.

Our study confirms the presence of a Pi-anion exchange mechanism across the BL membrane of proximal tubule-like cells (4, 7, 20). The fact that Pi efflux is accelerated by extracellular Pi and citrate, but not by Cl^-, lactate, succinate, or glutamate suggests that this anion transport system is specific. Oxidation or metabolism of citrate as well as that of succinate (21), added as Na^+ salts, results in net cell alkalinization, which was found to reduce rather than enhance ^{32}P efflux. Only citrate, but not succinate, another metabolized anion, promoted Pi efflux. Thus, it is likely that the effect of citrate is due to a specific direct trans-stimulation of Pi efflux rather than to the metabolism of citrate. Citrate is produced mostly by bone (22) and is utilized preferentially by kidneys (23, 24). Its plasma concentration is strongly influenced by the bone turnover rate, and thus, it may serve to link renal Pi reabsorption to bone accretion.

Based on the information described herein, it is reasonable to surmise that efflux of Pi across the BL cell membrane of OK cells occurs, in part, via an electroneutral Pi transporter capable of exchanging 2 moles of intracellular acidic Pi for each mole of extracellular basic Pi or citrate. Such system is stimulated by intracellular acidity and is inhibited by intracellular alkalinity. It is stimulated by extracellular sodium and may contribute to net basal-lateral Pi and acid extrusion from these cells.

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