

# The Role of Cytosolic Calcium in Chronic Adaptation to Phosphate Depletion in Opossum Kidney Cells\*

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Chronic dietary phosphate restriction is associated with up-regulation of sodium-dependent phosphate ( $\text{Na/P}_i$ ) cotransport by renal proximal tubular epithelial cells in association with increases in  $\text{Na/P}_i$  cotransporter mRNA and protein. We investigated whether changes in cytosolic calcium mediate this adaptive response in opossum kidney cells, a continuous line of renal epithelial cells. After 24 h of phosphate depletion, steady-state cytosolic calcium levels were increased; this increase was observed at physiologic levels of phosphate restriction and was prevented by the calcium channel blocker verapamil. Chronic phosphate depletion was also associated with parallel increases in  $\text{Na/P}_i$  cotransport activity,  $\text{Na/P}_i$  cotransporter mRNA, and  $\text{Na/P}_i$  cotransporter protein, all of which were blocked in verapamil-treated cells. Actinomycin D, at a dose that prevented the increase in  $\text{NaPi-4}$  mRNA during phosphate depletion, also prevented the increase in  $\text{Na/P}_i$  cotransport activity. Incubation with the calcium ionophore ionomycin or A23187 reproduced the increase in  $\text{Na/P}_i$  cotransporter mRNA in phosphate-replete cells. Conversely, chelation of cytosolic calcium by quin-2/AM prevented the increase in  $\text{Na/P}_i$  cotransporter mRNA in phosphate-depleted cells. The effect of an increase in cytosolic calcium was specific for the  $\text{Na/P}_i$  cotransporter as mRNA levels for the sodium-dependent glucose transporter were not affected. Our observations suggest that chronic phosphate restriction increases steady-state cytosolic calcium, which, in turn, increases transcription of  $\text{Na/P}_i$  cotransporter mRNA, thereby stimulating  $\text{Na/P}_i$  cotransport activity.

Chronic dietary  $\text{P}_i$  restriction results in widespread changes in the physiologic function of multiple cell types. These include impaired insulin secretion in pancreatic islet cells, impaired norepinephrine metabolism in synaptosomes, and impaired phagocytosis in polymorphonuclear cells (1–3).  $\text{P}_i$ -depleted cells also have elevated cytosolic calcium levels. *In vivo* administration of the calcium channel blocker verapamil to rats during chronic dietary  $\text{P}_i$  restriction prevents both the increase in cytosolic calcium as well as the characteristic physiologic changes (1–3). These observations suggest that an increase in cytosolic calcium is an important cellular mediator of many physiologic changes associated with phosphate restriction.

Chronic  $\text{P}_i$  deprivation is also associated with an increase in

$\text{P}_i$  reabsorption by the kidney, which is mediated by a specific, membrane-bound sodium-dependent phosphate ( $\text{Na/P}_i$ )<sup>1</sup> cotransporter in the proximal tubule (4, 5). This adaptive increase in  $\text{Na/P}_i$  cotransport activity is associated with parallel increases in the  $\text{Na/P}_i$  cotransporter mRNA and protein (6, 7), suggesting a transcriptional cellular mechanism. Moreover, recent studies suggest that changes in cytosolic calcium can affect mRNA levels by modulating gene transcription (8–10), providing a potential cellular mechanism for some of the observed effects of  $\text{P}_i$  depletion. On the basis of these previous investigations, we hypothesized that chronic  $\text{P}_i$  restriction increases cytosolic calcium in renal tubular epithelial cells and that this change, in turn, produces the observed increases in  $\text{Na/P}_i$  cotransporter mRNA, protein, and transport activity.

Opossum kidney (OK) cells, a continuous line of renal epithelial cells, are a useful experimental model for investigation of the cellular mechanisms involved in the adaptation to  $\text{P}_i$  restriction, in isolation from the multiple systemic changes associated with *in vivo*  $\text{P}_i$  deprivation (4, 5). The recent cloning of the OK cell  $\text{Na/P}_i$  cotransporter  $\text{NaPi-4}$  cDNA (11) permits quantification of the corresponding mRNA levels. In addition, we have raised a rabbit polyclonal antibody against  $\text{NaPi-4}$  protein (12) to enable direct measurements of  $\text{NaPi-4}$  protein levels. We have recently demonstrated in OK cells parallel increases in  $\text{Na/P}_i$  cotransport activity,  $\text{Na/P}_i$  cotransporter  $\text{NaPi-4}$  mRNA, and  $\text{NaPi-4}$  protein during chronic  $\text{P}_i$  restriction (12), similar to the changes observed in  $\text{P}_i$ -depleted rats (6, 7). Moreover, our preliminary studies detected an increase in cytosolic calcium in  $\text{P}_i$ -depleted OK cells. Using the newly available molecular tools and the  $\text{NaPi-4}$  antibody, we investigated whether an increase in cytosolic calcium might mediate the increases in  $\text{NaPi-4}$  mRNA,  $\text{NaPi-4}$  protein, and  $\text{Na/P}_i$  cotransport activity associated with chronic  $\text{P}_i$  restriction of OK cells. Specifically, we addressed three experimental questions. 1) Do maneuvers that prevent the increase in cytosolic calcium during  $\text{P}_i$  depletion (calcium channel blockers or calcium chelators) prevent the increase in  $\text{NaPi-4}$  mRNA? 2) Do other experimental maneuvers that increase cytosolic calcium in  $\text{P}_i$ -replete cells (calcium ionophores) also produce an increase in  $\text{NaPi-4}$  mRNA? 3) Are the effects of cytosolic calcium specific for  $\text{NaPi-4}$  mRNA?

## EXPERIMENTAL PROCEDURES

**Materials**—OK cells were a gift of Judith Cole (University of Missouri, Columbia, MO). Culture media and dishes were from Life Technologies, Inc. Fura-2/AM was from Teflabs (Austin, TX). The verapamil enantiomers *S*(-)-verapamil and *R*(+)-verapamil were from Research Biochemicals Inc. (Natick, MA). Enhanced chemiluminescent (ECL) kits for developing the Western blots were from Amersham (Buckinghamshire, United Kingdom). Peptide *N*-glycosidase F was from Boeh-

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<sup>1</sup> The abbreviations used are:  $\text{Na/P}_i$ , sodium-dependent phosphate; OK, opossum kidney; PCR, polymerase chain reaction; SGT, sodium-dependent glucose transporter.

ringer Mannheim. Multi-antigen peptides for antibody production were synthesized by Research Genetics (Huntsville, AL). PCR primers were synthesized by Operon Technologies, Inc. (Alameda, CA). All isotopes were from DuPont NEN. All other reagents were from Sigma.

**Cell Culture and Measurement of  $\text{Na}/\text{P}_i$  Cotransport**—OK cells were grown on 35-mm culture dishes in minimum essential medium with the addition of 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin. They were kept in an incubator at 37 °C with 95% air and 5%  $\text{CO}_2$ . 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.  $\text{P}_i$  transport was measured as previously reported by us (13). After reaching confluence, the cells were rendered quiescent by a 16-h incubation with serum-free minimum essential medium containing 1 mM  $\text{P}_i$  and 1% bovine serum albumin, followed by a 24-h incubation in serum-free medium containing normal or low  $\text{P}_i$  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  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 14.0 mM HEPES, and 0.1 mM  $\text{K}_2\text{H}^{32}\text{PO}_4$ , pH 7.4). The transport solution was aspirated; the cells were washed three times with ice-cold 137 mM NaCl and 14 mM HEPES, pH 7.4, to stop phosphate uptake; and  $^{32}\text{P}$  uptake was quantified by scintillation counting. Sodium-independent  $\text{P}_i$  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  $\text{P}_i$  uptake with and without sodium.

**Measurement of Cytosolic Calcium**—After the appropriate preincubation, the cells were detached with EDTA, resuspended in serum-free medium, and loaded with Fura-2/AM for 40 min at room temperature. They were washed and resuspended in balanced salt solution (127 mM NaCl, 3.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 5 mM glucose, and 10 mM HEPES, pH 7.4). The cells were transferred to 2-ml plastic cuvettes, continuously stirred at room temperature, and excited alternatively at wavelengths of 340 and 380 nm, with fluorescence emission measured at 510 nm, using a Photon Technology International Delta scanner. Cytosolic calcium was calculated from the ratio of emissions at the two excitation wavelengths, as previously validated in OK cells (14). The calcium channel blocker verapamil was used to prevent the increase in cytosolic calcium during  $\text{P}_i$  depletion. In addition, we used two biologically active enantiomers of verapamil: *S*(-)-verapamil, which also blocks calcium channels, and *R*(+)-verapamil, which does not (15). In some studies, the calcium ionophores ionomycin and A23187 were used to increase cytosolic calcium (16, 17). In other studies, the calcium chelator quin-2/AM was used to prevent an increase in cytosolic calcium (17). The medium was supplemented with 10 mM sodium pyruvate to prevent intracellular ATP depletion in the presence of quin-2/AM.

**Measurement of  $\text{Na}/\text{P}_i$  Cotransporter mRNA Levels**—After reverse transcription-PCR of OK cell RNA, forward (5'-TCC AGC ACA TC-TACC TCC ATC ATC-3') and reverse (5'-AGT GGG GTA ATG GCT-GAA GTG AAC-3') primers were used to synthesize a probe for NaPi-4 mRNA. The 0.8-kilobase PCR product was sequenced by a dye-labeled dideoxynucleotide sequencing kit (Applied Biosystems Inc.), and homology to the published NaPi-4 cDNA sequence (11) was confirmed. The NaPi-4 PCR fragment was radiolabeled with [ $^{32}\text{P}$ ]dCTP by random priming and hybridized with OK cell RNA using Northern analysis. Total RNA was isolated from OK cells by the guanidium isothiocyanate method (18), purified by phenol and chloroform extraction, precipitated with isopropyl alcohol, 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 at 42 °C with 5 × SSPE (sodium chloride/sodium phosphate/EDTA buffer), 2% SDS, and 50% formamide. After hybridization with the NaPi-4 probe, the membrane was washed twice at room temperature with 2 × SSPE and 0.1% SDS, followed by one wash at room temperature with 0.5 × SSPE and 0.2% SDS and one wash at 42 °C with 0.4 × SSPE and 0.2% SDS. The blots were exposed to Kodak X-AR film, and the bands were quantified by densitometry. NaPi-4 cDNA hybridized with Northern blots from OK cell RNA detected a single band at ~2.5 kilobases, comparable in size to NaPi-4 mRNA (11).

To evaluate whether  $\text{P}_i$  depletion nonspecifically induces the transcription of genes for other cell membrane-bound 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 (19) (at sites of homology to the rabbit and rat SGT cDNAs) were selected with the MacVector

software program and synthesized commercially. Following reverse transcription-PCR of OK cell RNA, this primer pair was used to make a ~600-base pair probe. Sequencing of this probe established >90% homology to the human SGT sequence. When this probe was hybridized with OK cell RNA Northern blots, it detected a single ~2.8-kilobase signal, representing OK cell SGT mRNA (12).

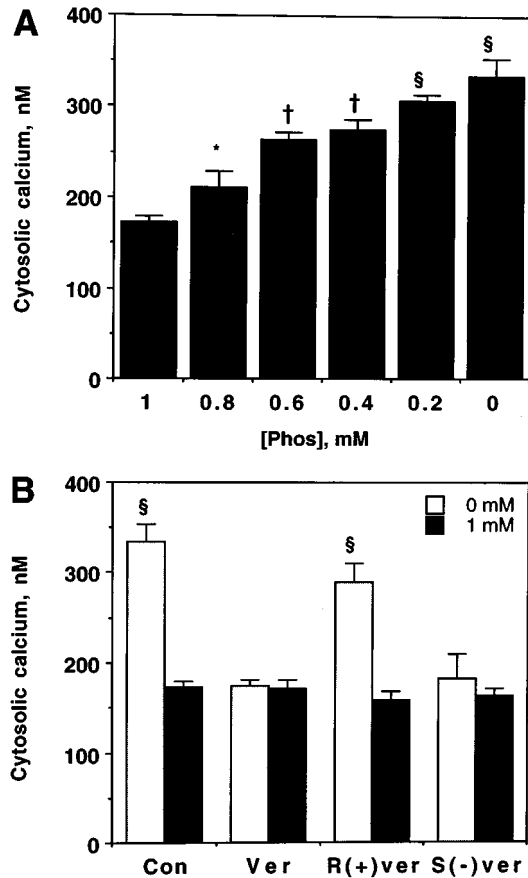
To provide a reference for total RNA per lane, the Northern blots were stripped and rehybridized with a probe for the glyceraldehyde-3-phosphate dehydrogenase "housekeeping gene." Degenerate forward (5'-AA(A/G)-TGG-GGT-GAT-GCT-GGT-GC(C/T)-G-3') and reverse (5'-CAT-GCC-AGT-GAG-(C/T)-TT-CCC-GTT-C-3') primer pairs for the rat, rabbit, and human glyceraldehyde-3-phosphate dehydrogenase cDNAs (20) were a kind gift of James Schafer (Department of Physiology, University of Alabama at Birmingham). After reverse transcription-PCR of OK cell RNA, these primers were used to synthesize a ~400-base pair probe. Sequencing of the PCR fragment revealed a ~90% homology to the published sequence for human glyceraldehyde-3-phosphate dehydrogenase cDNA. When this probe was hybridized with OK cell RNA Northern blots, it detected a single ~1.4-kilobase signal, representing the OK cell glyceraldehyde-3-phosphate dehydrogenase mRNA (12).

**Raising and Purification of Rabbit Polyclonal Anti-NaPi-4 Antibody**—The 8-amino acid peptide RSPARLPK (amino acids 566–573) from the published amino acid sequence of NaPi-4 (11) was found to have both high hydrophilic and antigenic indices, making it a promising epitope for the generation of antibodies. Multi-antigen peptides were synthesized, with the peptide coupled to polylysine to increase its immunogenicity. Rabbits were immunized with 1 mg of multi-antigen peptides suspended in Freund's complete adjuvant, followed by booster multi-antigen peptide injections suspended in Freund's incomplete adjuvant at 4 and 6 weeks. Blood samples obtained 10 days after the second booster revealed high antibody titers by enzyme-linked immunosorbent assay in the antisera of three of the four rabbits. The antibodies were precipitated from the antiserum with 50% ammonium sulfate saturation and centrifuged 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 three 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 Tris-buffered saline, pH 7.6. The 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-gauge needle. The resulting suspension was centrifuged (5 min at 1000 rpm). The supernatant was centrifuged for 30 min at 40,000 × *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  $\mu\text{M}$  leupeptin, 1  $\mu\text{M}$  soybean trypsin inhibitor, 0.1  $\mu\text{M}$  pepstatin, and 1  $\mu\text{M}$  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-polyacrylamide gel, and transferred to a nitrocellulose membrane. Non-specific 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. The purified rabbit polyclonal anti-NaPi-4 antibody detected an intense band at ~70 kDa, corresponding to the predicted size of NaPi-4 protein (11). 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 (data not shown), suggesting that it represents the glycosylated form of NaPi-4, analogous to the glycosylated form of the rat NaPi<sub>1</sub> cotransporter (NaPi-2) (21). Because the phosphate 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. An additional (weaker) band detected at ~51 kDa probably represents a degradation product of NaPi-4 protein.

## RESULTS

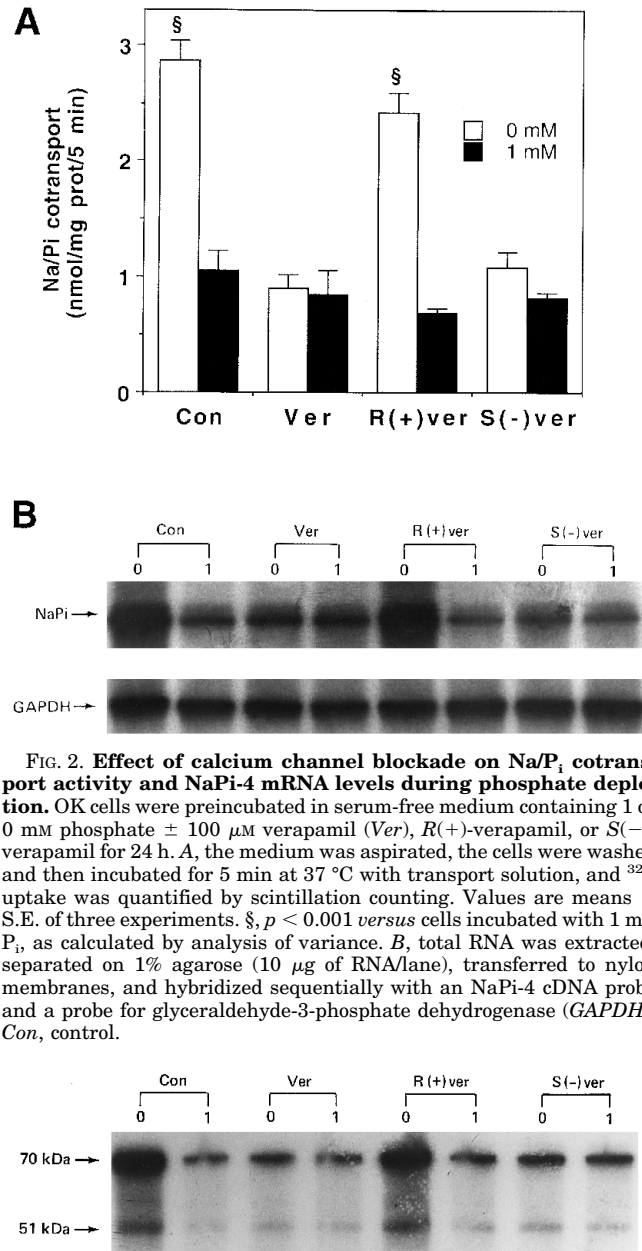
**Changes in Cytosolic Calcium during Phosphate Depletion**—Incubation of OK cells in a  $\text{P}_i$ -free medium for 24 h was associated with an approximate doubling of steady-state cytosolic calcium levels, as compared with  $\text{P}_i$ -replete cells (Fig. 1). This



**FIG. 1. Effect of phosphate restriction and verapamil on cytosolic calcium in OK cells.** OK cells were preincubated for 24 h in serum-free medium containing P<sub>i</sub> concentrations between 0 and 1 mM. The cells were then loaded with Fura-2/AM for 40 min, washed and resuspended in balanced salt solution, and excited alternatively at wavelengths of 340 and 380 nm, with fluorescence emission measured at 510 nm, using a Photon Technology International Delta scanner. Cytosolic calcium was calculated from the ratio of emissions at the two excitation wavelengths. *A*, the cells were incubated with medium containing 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM P<sub>i</sub> (Phos) prior to measurement of cytosolic calcium. *B*, the cells were incubated with the calcium channel blocker verapamil (Ver; 100  $\mu$ M) to prevent the increase in cytosolic calcium during P<sub>i</sub> depletion. In addition, we used two biologically active enantiomers of verapamil: *S*(-)-verapamil, which also blocks calcium channels, and *R*(+)-verapamil, which does not. Values are means  $\pm$  S.E. of three experiments. \*,  $p < 0.05$ ; †,  $p < 0.01$ ; §,  $p < 0.0001$  versus cells incubated with 1 mM P<sub>i</sub>, as calculated by analysis of variance. Con, control.

change was not limited to total P<sub>i</sub> depletion; rather, progressive P<sub>i</sub> restriction within the physiologic range was associated with progressive increases in cytosolic calcium (Fig. 1A). The calcium channel blocker verapamil prevented the increase in cytosolic calcium in P<sub>i</sub>-depleted cells, without affecting steady-state cytosolic calcium levels in cells incubated with 1 mM P<sub>i</sub> (Fig. 1B). *S*(-)-Verapamil reproduced the effect of verapamil on cytosolic calcium, whereas *R*(+)-verapamil did not, confirming the specificity of the former enantiomer for the calcium channel.

**Effect of Calcium Channel Blockade on Na/P<sub>i</sub> Cotransport Activity, NaPi-4 mRNA, and NaPi-4 Protein during Phosphate Depletion**—Incubation of OK cells in a 0 mM P<sub>i</sub> medium for 24 h was associated with a significant increase in Na/P<sub>i</sub> cotransport activity, as compared with cells incubated with 1 mM P<sub>i</sub> (Fig. 2A). Chronic P<sub>i</sub> depletion was also associated with parallel increases in NaPi-4 mRNA and protein, as compared with P<sub>i</sub>-replete cells (Figs. 2B and 3). Verapamil completely prevented the increases in NaPi-4 mRNA, NaPi-4 protein, and

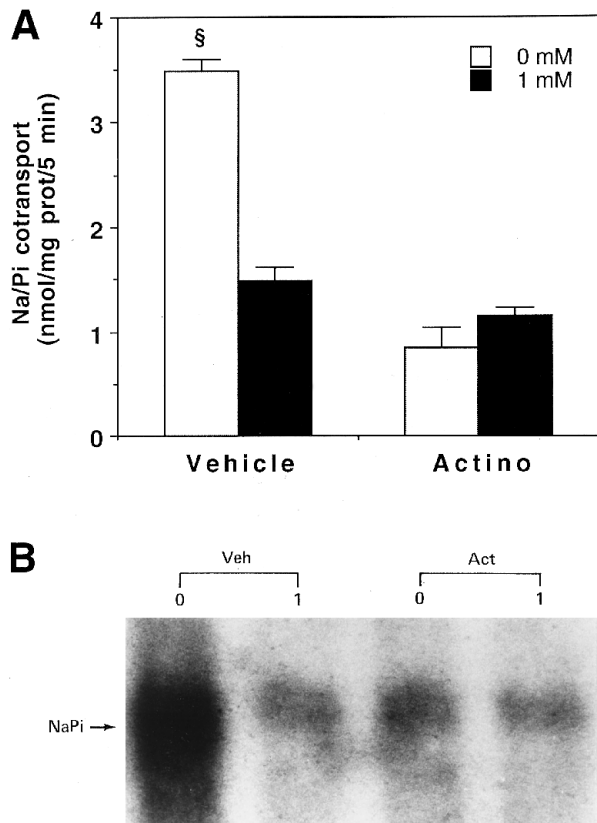


**FIG. 2. Effect of calcium channel blockade on Na/P<sub>i</sub> cotransport activity and NaPi-4 mRNA levels during phosphate depletion.** OK cells were preincubated in serum-free medium containing 1 or 0 mM phosphate  $\pm$  100  $\mu$ M verapamil (Ver), *R*(+)-verapamil, or *S*(-)-verapamil for 24 h. *A*, the medium was aspirated, the cells were washed and then incubated for 5 min at 37  $^{\circ}$ C with transport solution, and <sup>32</sup>P uptake was quantified by scintillation counting. Values are means  $\pm$  S.E. of three experiments. §,  $p < 0.001$  versus cells incubated with 1 mM P<sub>i</sub>, as calculated by analysis of variance. *B*, total RNA was extracted, separated on 1% agarose (10  $\mu$ g of RNA/lane), transferred to nylon membranes, and hybridized sequentially with an NaPi-4 cDNA probe and a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Con, control.

**FIG. 3. Effect of calcium channel blockade on NaPi-4 protein levels during phosphate depletion.** OK cells were preincubated with 1 or 0 mM P<sub>i</sub> medium  $\pm$  100  $\mu$ M verapamil (Ver), *R*(+)-verapamil, or *S*(-)-verapamil for 24 h. Crude membrane protein was extracted, deglycosylated with peptide *N*-glycosidase F, solubilized in SDS buffer, and boiled. The protein was run on SDS-polyacrylamide gel (10  $\mu$ g/lane) and transferred to a nitrocellulose membrane. After nonspecific binding with 5% nonfat dry milk, the membrane was incubated with rabbit anti-NaPi-4 antibody and then with goat anti-rabbit IgG conjugated to horseradish peroxidase and developed using the ECL kit. Con, control.

Na/P<sub>i</sub> cotransport activity in P<sub>i</sub>-depleted cells, without affecting any of these parameters in P<sub>i</sub>-replete cells. *S*(-)-Verapamil reproduced the inhibitory effects of verapamil, whereas *R*(+)-verapamil did not.

**Dependence of Adaptation to Phosphate Depletion on Gene Transcription**—Further experiments were performed to determine whether gene transcription was required for the chronic adaptation to phosphate depletion. Actinomycin D (0.25  $\mu$ g/ml), an inhibitor of DNA transcription, completely abolished the increase in Na/P<sub>i</sub> cotransport during chronic (24 h) P<sub>i</sub> depletion (Fig. 4A). Parallel Northern analysis confirmed that this dose of actinomycin prevented an increase in NaPi-4 mRNA levels



**FIG. 4. Effect of actinomycin on Na/Pi cotransport and NaPi-4 mRNA during chronic phosphate depletion of OK cells.** OK cells were incubated with 1 or 0 mM  $P_i$  for 24 h  $\pm$  actinomycin (*Actino*, *Act*; 0.25  $\mu$ g/ml). **A**, Na/Pi cotransport was measured after the incubation. Values are means  $\pm$  S.E. of four experiments.  $\$, p < 0.001$  versus 1 mM  $P_i$ , by Student's *t* test. **B**, total RNA was extracted, separated on 1% agarose (10  $\mu$ g of RNA/lane), transferred to nylon membranes, and hybridized with the NaPi-4 cDNA probe. *Veh*, vehicle.

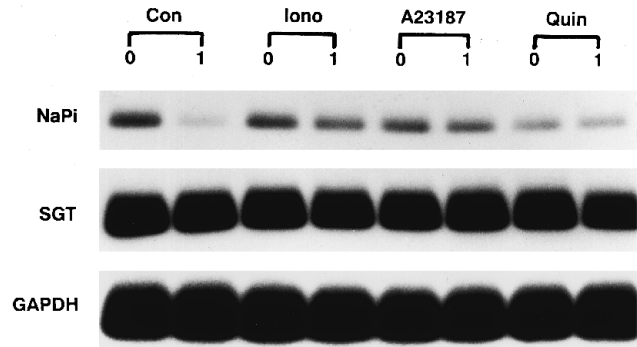
during  $P_i$  depletion (Fig. 4B).

**Effect of Calcium Ionophores on NaPi-4 mRNA**—Additional studies were performed to evaluate whether other experimental maneuvers that increase cytosolic calcium reproduce the effect of  $P_i$  depletion on the Na/Pi cotransporter mRNA. The calcium ionophores ionomycin and A23187 both increased NaPi-4 mRNA levels in OK cells incubated with 1 mM  $P_i$ , without affecting mRNA levels for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene (Fig. 5). Moreover, neither ionophore changed mRNA levels for an unrelated membrane-bound transporter, the sodium-dependent glucose transporter. Interestingly, there was no additive effect between  $P_i$  depletion and the calcium ionophores on NaPi-4 mRNA levels in OK cells.

**Effect of Calcium Chelators on NaPi-4 mRNA**—Additional studies demonstrated that the calcium chelator quin-2/AM could prevent the increase in NaPi-4 mRNA during  $P_i$  depletion of OK cells (Fig. 5). In contrast, the calcium chelator did not affect mRNA levels for the sodium-dependent glucose transporter.

#### DISCUSSION

Using OK cells, an experimental model for renal epithelial cells, we have demonstrated parallel increases in cytosolic calcium and Na/Pi cotransporter NaPi-4 mRNA during chronic (24 h) phosphate depletion. These observations suggest that an increase in cytosolic calcium mediates the increase in NaPi-4 mRNA in  $P_i$ -restricted OK cells. Alternatively,  $P_i$  depletion may increase cytosolic calcium and NaPi-4 mRNA by two in-



**FIG. 5. Effect of calcium ionophores and calcium chelators on NaPi-4 mRNA levels in  $P_i$ -depleted and  $P_i$ -replete OK cells.** The cells were incubated for 24 h in serum-free medium containing 1 or 0 mM  $P_i$   $\pm$  vehicle, ionomycin (*Iono*; 1  $\mu$ M), A23187 (1  $\mu$ M), or quin-2/AM (20  $\mu$ M). Total RNA was extracted, separated on 1% agarose (10  $\mu$ g of RNA/lane), transferred to nylon membranes, and hybridized sequentially with an NaPi-4 cDNA probe, a probe for SGT, and a probe for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *Con*, control.

dependent cellular mechanisms. To establish causality between the changes in cytosolic calcium and NaPi-4 mRNA, several conditions must be satisfied. First, prevention of the increase in cytosolic calcium should prevent the increase in NaPi-4 mRNA in  $P_i$ -depleted cells. Second, other experimental maneuvers that increase cytosolic calcium should also increase NaPi-4 mRNA. Finally, the effect of cytosolic calcium should be specific for NaPi-4 mRNA, without affecting other membrane-bound transporters.

The observed increase in cytosolic calcium in  $P_i$ -restricted OK cells is in agreement with similar findings in pancreatic islet cells, synaptosomes, and polymorphonuclear cells from rats (1–3). Chronic *in vivo*  $P_i$  deprivation is associated with multiple systemic changes (*e.g.* decreased parathyroid hormone and increased calcitriol and insulin), which may themselves affect cell function (4, 5). Because the increase in cytosolic calcium in this study occurred in cultured cells, in isolation from systemic factors, it must reflect an intrinsic effect of  $P_i$  restriction. Moreover, the finding of increased cytosolic calcium even during partial  $P_i$  restriction (Fig. 1A) establishes the physiologic significance of this effect. The cellular mechanism whereby  $P_i$  restriction increases cytosolic calcium in OK cells remains to be established. Levi *et al.* (2) have reported a decrease in the activity of the  $Ca^{2+}$ -ATPase in pancreatic islet cell membranes from  $P_i$ -restricted rats. This physiologic defect, by impairing cellular extrusion of calcium, would favor an increase in cytosolic calcium due to unopposed entry of calcium from the extracellular space.

Experimental maneuvers that impede entry of calcium into the cells or that chelate calcium may prevent an increase in cytosolic calcium in  $P_i$ -restricted cells. Thus, we have observed that incubation with the calcium channel blocker verapamil prevented the increase in NaPi-4 mRNA. Verapamil has multiple effects on cellular function that are independent of calcium channel blockade. Thus, for example, multiple immune functions of lymphocytes are inhibited by both *S*(-)-verapamil and *R*(+)-verapamil, even though only the former enantiomer blocks the calcium channel (15). Therefore, demonstrating inhibition of a particular cellular function by verapamil does not necessarily establish that cytosolic calcium mediates that function. In this study, however, the adaptations to  $P_i$  restriction were blocked only by *S*(-)-verapamil, and not by *R*(+)-verapamil. This discrepancy suggests that the increase in cytosolic calcium is in fact mediating the corresponding increases in Na/Pi cotransport activity, NaPi-4 mRNA, and NaPi-4 protein.

Even in the presence of a net influx of calcium into cells, a

calcium chelator prevents the anticipated increase in cytosolic calcium. We found that the calcium chelator quin-2/AM prevented the increase in NaPi-4 mRNA in  $P_i$ -restricted OK cells (Fig. 5). This observation adds further weight to the hypothesis that the increase in cytosolic calcium mediates the increase in NaPi-4 mRNA.

Calcium ionophores promote entry of calcium into the cell, also favoring an increase in cytosolic calcium. If the increase in cytosolic calcium mediates the increase in NaPi-4 mRNA in  $P_i$ -depleted cells, then calcium ionophores should increase NaPi-4 mRNA levels even in  $P_i$ -replete cells. Indeed, we found that incubation with two different calcium ionophores, ionomycin and A23187, reproduced the increase in NaPi-4 mRNA in  $P_i$ -replete OK cells (Fig. 5). Moreover, the effects of  $P_i$  restriction and the calcium ionophores on NaPi-4 mRNA were not additive, suggesting that both experimental maneuvers are mediated by a common biochemical pathway.

It is possible that an increase in cytosolic calcium produces multiple nonspecific cellular changes, rather than a specific increase in certain cellular functions. The lack of change in sodium-dependent glucose transporter mRNA during  $P_i$  restriction and calcium ionophore incubation (two experimental maneuvers that increase steady-state cytosolic calcium) suggests, however, that the effects are specific for the membrane-bound Na/ $P_i$  cotransporter.

The parallel increases in NaPi-4 mRNA, NaPi-4 protein, and Na/ $P_i$  cotransport activity in  $P_i$ -depleted OK cells are in agreement with our previous report (12). These observations suggest that the increase in NaPi-4 mRNA mediates the increase in Na/ $P_i$  cotransport activity during chronic  $P_i$  depletion. The prevention by actinomycin D of the increase in both NaPi-4 mRNA and Na/ $P_i$  cotransport activity during  $P_i$  depletion (Fig. 4) lends weight to this conclusion.

The observed increase in NaPi-4 mRNA during  $P_i$  depletion may reflect either an increase in transcription rate or an increase in mRNA stability. Our finding that actinomycin blocks the increase in NaPi-4 mRNA in  $P_i$ -restricted cells (Fig. 4) tends to support a transcriptional effect of cytosolic calcium. The recent demonstration of a direct modulation of transcription factors by calcium (8, 9) suggests that increased cytosolic calcium may interact with a transcription factor for NaPi-4, thereby increasing NaPi-4 mRNA levels. On the basis of our

experimental findings, we propose that phosphate depletion increases cytosolic calcium in renal tubular epithelial cells. The increase in cytosolic calcium modulates a transcription factor for the Na/ $P_i$  cotransporter gene. This effect, in turn, increases Na/ $P_i$  cotransporter mRNA and protein levels, ultimately up-regulating Na/ $P_i$  cotransport activity. Confirmation of this novel cellular mechanism will require a more detailed knowledge of the promoter and enhancer regions associated with the Na/ $P_i$  cotransporter DNA.

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