A phosphorylated phloretin derivative. Synthesis and effect on intestinal Na\(^{+}\)-dependent phosphate absorption

BRIAN E. PEERCE AND REBECCA CLARKE
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-0641

Received 10 July 2001; accepted in final form 2 November 2001

Peerce, Brian, E., and Rebecca Clarke. A phosphorylated phloretin derivative. Synthesis and effect on intestinal Na\(^{+}\)-dependent phosphate absorption. Am J Physiol Gastrointest Liver Physiol 283: G848–G855, 2002. First published November 7, 2001; 10.1152/ajpgi.00308.2001.—2’-Phosphophloretin (2’-PP), a phosphorylated derivative of the plant chalcone, was synthesized. The effect of 2’-PP, on Na\(^{+}\)-dependent phosphate uptake into intestinal brush-border membrane vesicles (BBMV) isolated from rabbit and rat duodenum and jejunum was examined. 2’-PP decreased Na\(^{+}\)-dependent phosphate uptake into rabbit BBMV with an IC\(_{50}\) of 55 nM and into rat BBMV with an IC\(_{50}\) of 58 nM. 2’-PP did not affect Na\(^{+}\)-dependent glucose, Na\(^{+}\)-dependent sulfate, or Na\(^{+}\)-dependent alanine uptake by rabbit intestinal BBMVs.

2’-PP inhibition of rabbit intestinal BBMV Na\(^{+}\)-dependent phosphate uptake was sensitive to external phosphate concentration, suggesting that 2’-PP inhibition of Na\(^{+}\)-dependent phosphate uptake was competitive with respect to phosphate. Binding of [\(^{3}H\)]2’-PP to rabbit intestinal BBMV was examined. Binding of [\(^{3}H\)]2’-PP was Na\(^{+}\)-dependent with a K\(_{D}\) for Na\(^{+}\) (Na\(^{+}\) concentration for 50% 2’-PP binding) of 30 mM. The apparent K\(_{D}\) for Na\(^{+}\)-dependent [\(^{3}H\)]2’-PP binding to rabbit BBMVs was 58 nM in agreement with the IC\(_{50}\) for 2’-PP inhibition of Na\(^{+}\)-dependent phosphate uptake. These results indicate that 2’-PP bound to rabbit or rat intestinal BBMV Na\(^{+}\)-phosphate cotransporter and inhibited Na\(^{+}\)-dependent phosphate uptake. In rats treated with 2’-PP by daily gavage, the effect of 2’-PP on serum phosphate, serum glucose, and serum calcium was examined. In a concentration-dependent manner, 2’-PP reduced serum phosphate by 45% 1 wk after starting treatment. 2’-PP did not alter serum calcium or serum glucose. The apparent IC\(_{50}\) for 2’-PP in vivo was 3 \(\mu\)M.

intestinal brush-border membrane vesicles; Na\(^{+}\)-phosphate cotransport; 2’-phosphophloretin; renal failure

SECONDARY HYPERPARATHYROIDISM is a common and severe complication of chronic renal failure resulting in renal osteodystrophy, hypertension, and metabolic acidosis (16, 18) and contributing to cardiac disease (22). Hyperphosphatemia due to decreased renal phosphate excretion is thought to contribute to secondary hyperparathyroidism in patients with chronic renal insufficiency (17, 32). Recently, it has been established that decreasing the phosphate load can reduce secondary hyperparathyroidism and possibly preserve renal function (18, 20, 21, 32, 37).

In mammals, intestinal phosphate absorption occurs at the brush-border membrane (BBM) in the proximal intestine (duodenum and jejunum). Phosphate absorption has active and passive components. Active uptake of phosphate is coupled to Na\(^{+}\) uptake down its electrochemical potential gradient by the Na\(^{+}\)-phosphate cotransporter. The active component of phosphate absorption is regulated by dietary phosphorus and serum 1,25-dihydroxy vitamin D\(_{3}\). Changes in dietary phosphorus have been reported to alter expression of NaP\(_{i}\) Ib in the intestine (13). Na\(^{+}\)-independent phosphate uptake occurs by an unknown mechanism down its electrochemical potential gradient. The mechanism of phosphate transport across the intestinal basolateral membrane has not been defined.

Effects of chalcones on membrane transport are well known. Phloridzin is a potent inhibitor of the renal and intestinal BBM Na\(^{+}\)-glucose cotransporters (9, 36). Phloretin, the aglucone of phloridzin, inhibits a variety of membrane transporters including Band 3 (AE-1) (9, 10) and the facilitated diffusion glucose carrier (GLUT-4) (9, 14). Unlike phloridzin, phosphorylated phloretin derivatives are not natural plant products. Starting with phloridzin, we have synthesized a phosphorylated derivative of phloretin, 2’-phosphophloretin (2’-PP). The effect of 2’-PP on intestinal BBM vesicle (BBMV) Na\(^{+}\)-dependent phosphate uptake was examined. 2’-PP was found to be a potent selective inhibitor of Na\(^{+}\)-dependent phosphate uptake in vitro. In a concentration-dependent manner 2’-PP inhibited Na\(^{+}\)-dependent phosphate uptake but not Na\(^{+}\)-independent phosphate uptake, Na\(^{+}\)-dependent sulfate uptake, nor Na\(^{+}\)-dependent alanine uptake into intestinal BBMV. Inhibition of Na\(^{+}\)-dependent phosphate uptake was sensitive to external phosphate, suggesting that 2’-PP competed for the phosphate-binding site on the intestinal Na\(^{+}\)-phosphate cotransporter.

The effect of 2’-PP on plasma phosphate, plasma calcium, and plasma glucose was examined in the rat.

Address for reprint requests and other correspondence: B. E. Peerce, University of Texas Medical Branch, 2200 Basic Science Bldg., 301 University Boulevard, Galveston, TX 77555-0641 (E-mail: BPeerce@UTMB.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Rats treated with 2'-PP by daily gavage were compared with rats treated with vehicle. In vivo 2'-PP decreased plasma phosphate but not plasma glucose or plasma calcium in a concentration-dependent manner. The apparent IC$_{50}$ was 3 μM.

**MATERIALS AND METHODS**

**Materials**

Salts and other chemicals used for the purification and assay of the BBMV were purchased from Fisher Chemical (Houston, TX). HEPES, Tris, MES, and PIPES were purchased from Sigma (St. Louis, MO). Chemicals and solvents used for the synthesis of 2'-PP, as well as phloridzin and phloretin, were purchased from Aldrich (Milwaukee, WI). [$^3$H]H$_2$O, [35S]sulfate, and [32P]phosphate were purchased from DuPont/NEN (Boston, MA). Membrane filters (0.45 μ) were purchased from Millipore (Boston, MA). Rat chow was purchased from Purina Mills, (Richmond, IN).

**Methods**

**BBMV.** Rabbit and rat intestinal BBMV were prepared by Ca$^{2+}$ precipitation and differential centrifugation from mucosal scraping of the proximal small intestine as previously described (24, 30). Purified BBMVs were stored in 300 mM dimethylformamide and 3.1 g potassium carbonate. Benzoic acid (Sigma-Aldrich) (2.7 ml) was added to this solution and the reaction was allowed to proceed for 3 days at 23°C. The solution was distilled under vacuum, and the residue was cooled to room temperature. Residue was extracted with water/ethyl acetate (2:1) three times. The organic layers were combined and concentrated by roto evaporation. Residue was dissolved in 1.4 dioxane (200 ml), and HCl was added to a final concentration of 0.4 N. The mixture was refluxed for 3 h. The reaction mixture was cooled, diluted with 1 M sodium bicarbonate, and extracted with ethyl acetate. Ethyl acetate extraction was repeated three times. The combined organic layers were washed with water, followed by two washes with NaCl (0.9%). Anhydrous Na$_2$SO$_4$ was added to the organic layer and mixed. Vacuum filtration removed the Na$_2$SO$_4$. Fresh Na$_2$SO$_4$ was added to the solution, which was then stirred for 12 h at 23°C. Vacuum filtration removed the Na$_2$SO$_4$, and the filtrate was concentrated to yield 4',6,4-tri-O-benzylphloretin (2.1 g, 92% yield); $^3$H-NMR (CDCl$_3$) δ 13.6 [singlet (s), 1H]; 7.46–7.29 [multiple (m), 15H]; 6.86 [doublet (d), coupling constant ($\text{J}$) = 8.8 Hz, 2H]; 6.8 (d, $\text{J}$ = 8.8 Hz, 2H); 6.35 (d, $\text{J}$ = 2.3 Hz, 1H); 6.21 (d, $\text{J}$ = 2.3 Hz, 1H); 5.17 (s, 2H); 5.14 (s, 2H); 5.07 (s, 2H); 3.2 [triplet (t), $\text{J}$ = 7.1 Hz, 2H].

**Synthesis of 2'-PP.** Method 1. Phlorizin (2 g) was added to acetic anhydride (20 ml) and sodium acetate (1.6 g) and was heated at 100°C for 6 h. Reactants were cooled, and crystaline phloridzin hepta-acetate was recovered by filtration. Crystals were dissolved in 50 ml of hot methanol and hot filtered (hot water jacketed funnel). The hepta-acetate (1.2 g, 34% yield) was recovered.

The hepta-acetate (0.6 g) was added to 0.4 N H$_2$SO$_4$ (100 ml), and heated to reflux. The reaction was allowed to proceed for 3 h. Reactants were cooled, washed with methanol, and filtered. Phloretin triacetate (0.3 g, 92% yield) was recovered.

Phloretin triacetate was phosphorylated with dry phosphoric acid, which was dried by the addition of phosphoric acid (8.5 ml of 85% phosphoric acid) to phosphorus pentoxide (5 g) during 1 h. Phloretin triacetate was added to the solution, and the reaction was allowed to proceed under reduced pressure for 5 days.

After phosphorylation, the acetate groups were removed by dilution of the phosphophloretin triacetate with potassium carbonate until the pH of the solution was between 22- and 35-fold. During the course of the experiments BBMV protein enrichment varied between 24- and 30-fold in the brush-border enzyme markers. Intestinal homogenate and BBMV protein were assayed by the method of Bradford using fluorescamine (11) and sucrase (7). Intestinal homogenate and BBMV protein enrichment varied between 22- and 35-fold in the brush-border enzyme markers.

**Assignment of the structure of the inhibitor shown in Fig. 10, 2'-PP, was based on comparison with the $^1$H-NMR and 13C-NMR spectra of phloridzin (9, 29) and standard chemical shift tables (31). The NMR results were compared with $^{13}$NMR and 13C-NMR spectra of phloretin and phloridzin.
using the NMR Predictor software program, Advanced Chemistry Development (Ottawa, Canada).

Synthesis of \[^{3}H\]2-PP. 2-PP (10 mg) was labeled with tritium by catalytic exchange of ring protons by \[^{3}H_{2}O\] as previously described (12, 33). The product was purified by thin-layer chromatography on silica gel plates developed with 70% chloroform/30% methanol (9). Labeled 2-PP was identified by comparison with authentic 2-PP run in parallel. The specific activity of \[^{3}H\]2-PP was 12 Ci/mmol.

\(Na^+\)-dependent phosphate uptake. \(Na^+\)-dependent phosphate uptake was determined by a rapid mixing and filtration procedure using 100 \(\mu\)g of BBMV protein, 3-s uptakes, and filtration through 0.45-\(\mu\) filters (34). \(Na^+\)-dependent uptake was defined as uptake in the presence of 100 mM NaCl, 100 mM mannitol, 100 \(\mu\)M \[^{32}P\]phosphate, and 10 mM HEPES/Tris, pH 7.5. Uptakes were performed for 5 s at 23°C in the presence of 100 mM KCl, 100 mM mannitol, 100 \(\mu\)M \[^{32}P\]phosphate, and 10 mM HEPES/Tris, pH 7.5. All determinations were performed in triplicate. Filter-retained counts were determined by liquid scintillation counting and are reported as pmoles per mg protein second.

Experiments examining the effect of 2-PP or phloretin on \(Na^+\)-dependent phosphate uptakes were performed by membrane filtration. 2-PP was diluted with 10 mM KOH/borate buffer, pH 7, and added to the uptake tube immediately before the start of the experiment. 2-PP concentration was varied between 10 nM and 10 \(\mu\)M. Experiments examining the effect of 2-PP on \(Na^+\)-dependent glucose uptake or \(Na^+\)-dependent alanine uptake were performed as described above. 2-PP was added immediately before the start of the experiment. \(Na^+\)-dependent glucose uptake was defined as uptake in the presence of 100 mM NaCl, 100 mM mannitol, 50 \(\mu\)M \[^{3}H\]glucose, and 10 mM HEPES/Tris, pH 7.5, minus uptake in the presence of 100 mM KCl, 100 mM mannitol, 50 \(\mu\)M \[^{3}H\]glucose, and 10 mM HEPES/Tris, pH 7.5. \(Na^+\)-dependent alanine uptake was defined as uptake in the presence of 100 mM NaCl, 100 mM mannitol, 100 \(\mu\)M \[^{3}H\]alanine, and 10 mM HEPES/Tris, pH 7.5, minus uptake in the presence of 100 mM KCl, 100 mM mannitol, 100 \(\mu\)M \[^{3}H\]alanine, and 10 mM HEPES/Tris, pH 7.5. All determinations were performed in triplicate.

Experiments examining the effect of 2-PP on \(Na^+\)-dependent intestinal BBMV were performed as previously described (1, 23). \(Na^+\)-dependent sulfate uptake was defined as BBMV uptake of 1 mM \[^{35}S\]sulfate in the presence of 100 mM NaCl, 100 mM mannitol, and 10 mM HEPES/Tris, pH 7.5, minus uptake of 1 mM \[^{35}S\]sulfate in 100 mM KCl, 100 mM mannitol, and 10 mM HEPES/Tris, pH 7.5. Uptakes were performed for 5 s at 23°C.

\(\Delta\)Binding of \[^{3}H\]2-PP to BBMV protein. Binding of \[^{3}H\]2-PP to intestinal BBMV protein was determined by membrane filtration as described for phosphate uptake by BBMV protein. \(Na^+\)-dependent \[^{3}H\]2-PP bound to BBMV protein was measured after a 60-s incubation of 100 \(\mu\)g of BBMV protein with 100 mM NaCl, 10 mM PIPES/Tris, pH 7, 100 mM mannitol, and 10 mM to 10 \(\mu\)M \[^{3}H\]2-PP, or with 100 mM KCl, 100 mM mannitol, 10 mM PIPES/Tris, pH 7, and 10 nM to 10 \(\mu\)M 2-PP at 4°C. After 1 min, the reaction mixture was diluted 10 times with (in mM): 100 NaCl, 100 mannitol, and 10 PIPES/Tris, pH 7 (NaCl wash solution), or 100 KCl, 100 mannitol, and 10 PIPES/Tris, pH 7 (KCl wash solution), and filtered through 0.45-\(\mu\) filters. Filters were washed with an additional 5 ml of the appropriate wash solution. Filter-retained counts were determined by liquid scintillation counting. \(Na^+\)-dependent 2-PP binding was defined as filter-retained counts in the presence of 100 mM NaCl, 100 mM mannitol, and 10 mM PIPES/Tris, pH 7, minus filter-retained counts in the presence of 100 mM KCl, 100 mM mannitol, and 10 mM PIPES/Tris, pH 7.

In some experiments, BBMV protein was labeled with the arginine reagent phenylglyoxal before labeling with 2-PP. In these experiments, 5 mg of BBMV protein was labeled with 2 mM phenylglyoxal in 300 mM mannitol and 20 mM HEPES/Tris, pH 7.5, for 30 min at 23°C (25). The reaction was stopped by 10-fold dilution with ice-cold 300 mM mannitol and 20 mM HEPES/Tris, pH 7.5. Protein concentrations were determined by the SDS Micro-Lowry method (28) on an aliquot of protein taken from the phenylglyoxal-labeled protein. \(Na^+\)-dependent \[^{3}H\]2-PP binding was determined as described above. Protein was corrected for losses during phenylglyoxal labeling.

In some experiments, the effect of external substrates on \[^{3}H\]2-PP binding was examined. Experiments examining the effect of NaCl on \[^{3}H\]2-PP binding varied external NaCl between 10 and 150 mM during incubation with \[^{3}H\]2-PP at 4°C. In experiments examining the effect of NaCl concentration on \[^{3}H\]2-PP binding, osmotic strength was maintained at 300 mosM by the addition of mannitol in exchange for NaCl.

In some experiments, the effect of external phosphate on \[^{3}H\]2-PP binding was examined. In these experiments, 250 \(\mu\)M potassium phosphate was added during the incubation with 2-PP at 4°C.

In vivo studies. Adult rats were fed Purina Mills Test Diet 5575 containing 0.6% calcium and 0.6% phosphorus. Animals were maintained on this diet for 3 wk before starting the experiment. Rats were bled from the tail 14 and 7 days before the start of the experiment. Rats were dosed daily with varying amounts of 2-PP in 3 ml of 150 mM NaCl and 10 mM PIPES/Tris, pH 7, by gavage. Control rats were dosed with 3 ml of 150 mM NaCl and 10 mM PIPES/Tris, pH 7. Blood was drawn before the second treatment with 2-PP and every 3rd day, thereafter. Plasma was separated and stored at −20°C. Plasma calcium and glucose were determined spectrophotometrically using clinical kits from Sigma. Serum phosphate was measured as phosphorus using a clinical kit from Sigma. Determinations were performed in duplicate and the results are expressed as means ± SE.

RESULTS

Effect of 2-PP on \(Na^+\)-Dependent Phosphate Uptake by Intestinal Apical Membrane Vesicles

The effect of 2-PP on phosphate uptake into rabbit intestinal BBMV is shown in Fig. 1. In the presence of \(Na^+\), 2-PP inhibited phosphate uptake into rabbit intestinal BBMV in a 2-PP concentration-dependent manner. In the absence of \(Na^+\), 2-PP did not affect phosphate uptake into rabbit intestinal BBMV. \(Na^+\)-dependent phosphate uptake is the difference between phosphate uptake in the presence of NaCl and phosphate uptake in the presence of KCl.

Specificity of intestinal BBMV \(Na^+\)-dependent phosphate uptake for the phosphorylated derivative of phloretin is shown in Fig. 2. In a concentration-dependent manner, 2-PP inhibited \(Na^+\)-dependent phosphate uptake. The apparent IC\(_{50}\) for 2-PP inhibition of \(Na^+\)-dependent phosphate uptake was 55 ±
6 nM (n = 15 experiments). Phloretin had no effect on Na\(^+\)-dependent phosphate uptake. Na\(^+\)-dependent phosphate uptake into rabbit BBMV was inhibited 5 ± 3% (n = 5) at 10 mM phloretin.

Similar results were seen for 2'-PP inhibition of rat BBMV Na\(^+\)-dependent phosphate uptake. The apparent IC\(_{50}\) for 2'-PP inhibition of Na\(^+\)-dependent phosphate uptake was 58 ± 9 nM (n = 4). Na\(^+\)-dependent phosphate uptake was insensitive to 2'-PP. Na\(^+\)-dependent phosphate uptake into rat BBMV was insensitive to 10 μM phloretin (results not shown).

**Effect of 2'-PP on other Na\(^+\)-Dependent Cotransporters**

Specificity of 2'-PP for the rabbit Na\(^+\)-phosphate cotransporter was examined by testing the effect of 2'-PP on Na\(^+\)-dependent glucose uptake and Na\(^+\)-dependent alanine uptake into intestinal BBMV. Results of these studies are shown in Fig. 3. Na\(^+\)-dependent glucose uptake and Na\(^+\)-dependent alanine uptake were not affected by 2'-PP at 2'-PP concentrations 50 times that required for 90% inhibition of Na\(^+\)-dependent phosphate uptake. These results are consistent with 2'-PP being a specific inhibitor of the intestinal Na\(^+\)-phosphate cotransporter.

The effect of 2'-PP on Na\(^+\)-dependent sulfate uptake into rabbit BBMV is shown in Fig. 4. Na\(^+\)-dependent sulfate uptake was not affected by 2'-PP concentrations ≤10 μM. Na\(^+\)-dependent phosphate uptake is shown for comparison.

---

**Methods**

In all experiments, vesicles were prepared according to the method of Hokin and coworkers (1981). Phosphate uptake was performed as described in Methods, and the amount of filter-retained counts was determined by liquid scintillation counting. Na\(^+\)-dependent phosphate uptake was defined as uptake in the presence of 100 mM NaCl minus uptake in the presence of 100 mM KCl. Results are means ± SE of triplicate determinations and representative of 3 separate experiments.
dependent phosphate uptake consistent with competitive inhibition by 2’-PP. The $K_i$ for 2’-PP was determined from the point of intersection of the lines of the Dixon plot. The $K_i$ in rabbit BBMV was $38 \pm 7$ nM ($n = 4$). Similar experiments in rat BBMV resulted in a $K_i$ for 2’-PP of $42$ nM $\pm 8$ nM ($n = 3$).

$[^3H]2'$-PP Binding to Intestinal BBMV Protein

Binding of $[^3H]2'$-PP to intestinal BBMV protein is shown in Fig. 6. In the absence of Na$^+$, $1.2 \pm 0.3$ pmoles of 2’-PP/mg protein bound to intestinal BBMV protein. In the presence of Na$^+$, $10.3 \pm 0.9$ pmoles of 2’-PP/mg protein bound to intestinal BBMV. Pretreatment of the intestinal BBMV with the arginine reagent phenylglyoxal decreased Na$^+$-dependent 2’-PP labeling to $1.5 \pm 0.2$ pmoles of 2’-PP/mg protein (results not shown).

2’-PP binding was also examined as a function of Na$^+$ concentration and phosphate concentration. The apparent $K_{0.5}$ for Na$^+$ was $30 \pm 4$ mM ($n = 3$ rabbits). The addition of 250 $\mu$M phosphate during $[^3H]2'$-PP binding reduced Na$^+$-dependent $[^3H]2'$-PP binding to $1.4 \pm 0.4$ pmoles/mg protein ($n = 3$ experiments).

Figure 7 is a Scatchard plot analysis of Na$^+$-dependent $[^3H]2'$-PP binding to rabbit intestinal BBMV. Results of 2’-PP binding to intestinal BBMV protein were fit to a straight line. The intrinsic dissociation constant for 2’-PP, $K_d$, was determined from the slope of the Scatchard plot. The $K_d$ for 2’-PP binding to rabbit BBMV was $51 \pm 8$ nM ($n = 3$). The $K_d$ for 2’-PP binding is consistent with the apparent $IC_{50}$ of 2’-PP inhibition of Na$^+$-dependent phosphate uptake (Figs. 1 and 2).

In Vivo Studies

The effect of 2’-PP on plasma phosphate was examined in rats. The effect of 2’-PP on plasma phosphate,

**Effect of Substrates of the Na$^+$-Phosphate Cotransporter on 2’-PP Inhibition of Na$^+$-Dependent Phosphate Uptake**

The effect of external phosphate on 2’-PP inhibition of Na$^+$-dependent phosphate uptake into rabbit BBMV was also examined. Results of these studies are shown in Fig. 5.

Figure 5 is a Dixon plot showing inhibition of Na$^+$-dependent phosphate uptake by 10 nM to 1 $\mu$M 2’-PP at 50 $\mu$M phosphate, 100 $\mu$M phosphate, and 250 $\mu$M phosphate. The Dixon plot indicates that increasing external phosphate decreased 2’-PP inhibition of Na$^+$-dependent phosphate uptake consistent with competitive inhibition by 2’-PP. The $K_i$ for 2’-PP was determined from the point of intersection of the lines of the Dixon plot. The $K_i$ in rabbit BBMV was $38 \pm 7$ nM ($n = 4$). Similar experiments in rat BBMV resulted in a $K_i$ for 2’-PP of $42$ nM $\pm 8$ nM ($n = 3$).

$[^3H]2'$-PP Binding to Intestinal BBMV Protein

Binding of $[^3H]2'$-PP to intestinal BBMV protein is shown in Fig. 6. In the absence of Na$^+$, $1.2 \pm 0.3$ pmoles of 2’-PP/mg protein bound to intestinal BBMV protein. In the presence of Na$^+$, $10.3 \pm 0.9$ pmoles of 2’-PP/mg protein bound to intestinal BBMV. Pretreatment of the intestinal BBMV with the arginine reagent phenylglyoxal decreased Na$^+$-dependent 2’-PP labeling to $1.5 \pm 0.2$ pmoles of 2’-PP/mg protein (results not shown).

2’-PP binding was also examined as a function of Na$^+$ concentration and phosphate concentration. The apparent $K_{0.5}$ for Na$^+$ was $30 \pm 4$ mM ($n = 3$ rabbits). The addition of 250 $\mu$M phosphate during $[^3H]2'$-PP binding reduced Na$^+$-dependent $[^3H]2'$-PP binding to $1.4 \pm 0.4$ pmoles/mg protein ($n = 3$ experiments).

Figure 7 is a Scatchard plot analysis of Na$^+$-dependent $[^3H]2'$-PP binding to rabbit intestinal BBMV. Results of 2’-PP binding to intestinal BBMV protein were fit to a straight line. The intrinsic dissociation constant for 2’-PP, $K_d$, was determined from the slope of the Scatchard plot. The $K_d$ for 2’-PP binding to rabbit BBMV was $51 \pm 8$ nM ($n = 3$). The $K_d$ for 2’-PP binding is consistent with the apparent $IC_{50}$ of 2’-PP inhibition of Na$^+$-dependent phosphate uptake (Figs. 1 and 2).

In Vivo Studies

The effect of 2’-PP on plasma phosphate was examined in rats. The effect of 2’-PP on plasma phosphate,
plasma calcium, and plasma glucose in 16- to 18-mo-old rats is shown in Fig. 8. $2'$-PP (10 $\mu$M) reduced plasma phosphate 45% ($n = 4$) within 4 days after the start of treatment. Plasma Ca$^{2+}$ and plasma glucose were unaffected by $2'$-PP.

Effects of $2'$-PP concentration on plasma phosphate, and on plasma calcium after 7 days of daily gavage are shown in Fig. 9. Plasma phosphate decreased 42% compared with vehicle-treated controls. Plasma calcium was not affected by $2'$-PP administered daily. $2'$-PP concentration resulting in half of the maximal effect on plasma phosphate was 3.2 $\pm$ 0.4 $\mu$M ($n = 3$ rats).

DISCUSSION

A potential inhibitor of the intestinal BBM Na$^+$-phosphate cotransporter was synthesized and examined for its effect on intestinal phosphate absorption. The proposed structure of the compound $2'$-PP is shown in Fig. 10.

The addition of $2'$-PP to intestinal rabbit BBMV (Figs. 1 and 2) or rat intestinal BBMV decreased Na$^+$-dependent phosphate uptake with an IC$_{50}$ of 55 nM in rabbit BBMV and 58 nM in rat BBMV. The observed inhibition was >90% at $2'$-PP concentrations above 100 nM. The effect of $2'$-PP was specific for Na$^+$-dependent phosphate uptake. $2'$-PP did not alter Na$^+$-independent phosphate uptake (Fig. 1), Na$^+$-dependent glucose uptake (Fig. 3), or Na$^+$-dependent alanine uptake (Fig. 3). $2'$-PP did not inhibit Na$^+$-dependent sulfate uptake into rabbit intestinal BBMV (Fig. 4). This result is in contrast with the effect of phosphonoformic acid on Na$^+$-dependent phosphate uptake into intestinal BBMV (19).

$2'$-PP inhibition of Na$^+$-dependent phosphate uptake in BBMV appeared to be competitive with respect to external phosphate. Increasing the concentration of external phosphate from 50 to 250 $\mu$M reduced $2'$-PP
inhibition of Na\(^+\)-dependent phosphate uptake (Fig. 5). The effect of external phosphate on \(^{3}H\)2'-PP binding to intestinal BBMV is consistent with this interpretation.

Binding of 2'-PP to intestinal BBMV protein as measured by \(^{3}H\)2'-PP binding was Na\(^+\)-dependent. In a concentration-dependent manner, Na\(^+\) enhanced 2'-PP binding to rabbit intestinal BBMV. The Na\(^+\) concentration resulting in \(K_{0.5}\) was 30 mM, similar to the Na\(^+\) concentration resulting in 50% activation of phosphate uptake (3, 8, 27), and the \(K_{0.5}\) for the Na\(^+\)-induced conformational change (26). Binding of 2'-PP to intestinal BBMV was decreased by the addition of external phosphate and was inhibited by covalent labeling of the arginine reagent phenylglyoxal. Phenylglyoxal has been reported to inhibit intestinal Na\(^+\)-dependent phosphate uptake in a Na\(^+\)/phosphate-sensitive manner (24, 25) and to label a 120-kDa polypeptide in an Na\(^+\) + phosphate-sensitive manner (25). These results are consistent with 2'-PP binding to the intestinal Na\(^+\)-phosphate cotransporter at the cotransporter phosphate site (3, 25–27). Results of the in vitro studies with 2'-PP strongly suggest that 2'-PP is a specific inhibitor of the intestinal BBM Na\(^+\)-phosphate cotransporter.

In vivo, the effect of 2'-PP was examined in aged adult rats by using plasma phosphate concentration as a measure of the efficacy of 2'-PP. It is well known that 1,25-dihydroxy vitamin D\(_3\) upregulates intestinal calcium and phosphate absorption (2, 4, 15, 17). Aged adult rats were selected for these studies to take advantage of the age-related reduction in serum levels of 1,25-dihydroxy vitamin D\(_3\) and decreased intestinal responsiveness to 1,25-dihydroxy vitamin D\(_3\).

Plasma phosphate was sensitive to 2'-PP. In a time-dependent and 2'-PP concentration-dependent manner, 2'-PP reduced plasma phosphate 45% during the course of 2-wk trials where 2'-PP was administered by daily gavage (Figs. 8 and 9). The effect of 2'-PP in vivo was specific for plasma phosphate. Serum calcium and serum glucose were not affected by 2'-PP (Fig. 8). The effect of 2'-PP on plasma phosphate was 2'-PP concentration dependent. The apparent 2'-PP concentration resulting in a 50% effect on plasma phosphate was 3 \(\mu\)M.

In vivo 2'-PP studies did not show a significant change in plasma calcium as is commonly seen when animals are placed on a drastically reduced phosphorus diet. It is common for these animals to become hypercalcemic due to the drastic reduction in plasma phosphate. With only the intestinal Na\(^+\)-phosphate cotransporter contribution to intestinal phosphate absorption affected by 2'-PP, there was <50% reduction in plasma phosphate. This change in plasma phosphate may represent a subthreshold change in plasma phosphate, especially in aged rats.

A pharmaceutical approach to reducing serum phosphate and serum parathyroid hormone in chronic renal insufficiency patients would be a major advance in treatment of this disease. A number of candidates have been tested, including phosphonofumaric acid (6, 19) and vitamin D derivatives (35, 38). Studies described here suggest that 2'-PP or a similar compound may have promise as a therapy in chronic renal failure.

The authors thank Edward Ezell, Sealy Center for Structural Biology, University of Texas Medical Branch, Galveston, TX, for doing the NMR experiments and for his expert assistance in NMR analysis of 2'-PP.

REFERENCES