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Inhibition of human intestinal brush border membrane vesicle Na^+-dependent phosphate uptake by phosphophloretin derivatives

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Abstract

Hyperphosphatemia and II° hyperparathyroidism are common and severe complications of chronic renal failure. Reduced dietary phosphorus has been shown to be an effective treatment in reducing serum phosphate and serum PTH. 2′-Phosphophloretin inhibited small intestine apical membrane Na^+/phosphate cotransport and reduced serum phosphate in adult rats. 2′-PP and phosphoesters of phloretin were tested for inhibition of human small intestine brush border membrane alkaline phosphatase activity and for inhibition of Na^+-dependent phosphate uptake. The IC_{50}'s for inhibition of alkaline phosphatase suggested an order of inhibitory potency of 4-PP > phloretin > 4′-PP > 2′-PP. Inhibition of Na^+-dependent phosphate uptake followed the sequence 2′-PP ≫ 4′-PP > 4-PP > phloretin. These results are consistent with 2′-PP being a specific inhibitor of human intestinal brush border membrane Na^+/phosphate cotransport.

Keywords: Human intestinal brush border membrane; Intestinal phosphate absorption; Na^+-dependent phosphate uptake; Na^+/phosphate cotransporter; 2′-Phosphophloretin

In chronic renal failure phosphate retention and deposition as calcium phosphate precipitates contribute to interstitial injury, renal tubule injury, and cardiac disease [1–4]. Very low phosphorus diets in combination with phosphate binding compounds have been shown to slow the progression of renal failure. A pharmacological method of reducing intestinal phosphate absorption may provide a more palatable approach to reducing serum phosphate and may slow the progression of moderate chronic renal failure to end-stage renal failure.

In the proximal small intestine phosphate absorption occurs by a Na^+-dependent mechanism and a Na^+-independent process. Na^+-dependent phosphate uptake occurs through the Na^+/phosphate cotransporter. The Na^+/phosphate cotransporter has been identified as a 110–120 kDa polypeptide [5–8]. The mechanism of Na^+-independent uptake is unknown.

A phosphate ester of phloretin has been shown to inhibit rat and rabbit intestinal brush border membrane vesicle Na^+-dependent phosphate uptake [9]. 2′-PP inhibition of brush border membrane (BBM) Na^+-dependent phosphate uptake required Na^+ and was sensitive to external phosphate. In vivo 2′-PP reduced plasma phosphate in rats in a concentration-dependent manner. We have extended our studies of the effect of phosphophloretins to human BBM alkaline phosphatase activity and phosphate uptake into human BBM vesicles.

Materials and methods

Materials. Chemicals used in the synthesis of 2′-PP, 4′-PP, and 4-PP were purchased from Aldrich Chemical, Milwaukee, WI. 3-(4-hydroxyphenyl)-propionitrile was purchased from Lancaster Chemical, Lancaster, PA. All organic solvents were purchased from Aldrich Chemical, Milwaukee, WI and were of reagent grade or better. Membrane filters were purchased from Millipore, Boston, MA. [^{32}P]Phosphate was purchased from DuPont/NEN, Wilmington, DE. Salts and

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reagents used in the preparation and assay of brush border membrane vesicles were purchased from Fisher Chemical, Houston, TX.

Methods

Preparation of brush border membrane vesicles. Human intestine removed during surgical procedures was scraped and the mucosa was stored in 300 mM mannitol and 10 mM Hepes/Tris, pH 7.5, at liquid N_2 temperatures until needed. Brush border membrane vesicles were prepared by Ca^{2+} precipitation and differential centrifugation as previously described [5,6,10–15]. Purification of brush border membranes was assayed using the brush border membrane enzyme markers sucrase [16] and alkaline phosphatase [17]. During the course of these studies, enrichment in brush border membrane enzymes varied between 20- and 28-fold.

Synthesis of phosphophloretin derivatives. 2′-Phosphophloretin (2′-PP) was synthesized from phloridzin [9]. 2′-PP was analyzed by Mass Spectrometry, ^{31}P NMR ^{13}CNMR, and ^{1}HNMR [9]. ^{1}H NMR (400 Hz, d_6-DMSO) δ 13.0 (s, 1H), 10.7 (br. s, 1H), 9.2 (br. s., 1H), 7.03 (d, $J = 8.6$ Hz, 2H), 6.64 (d, $J = 8.4$ Hz, 2H), 6.63 (dd, $J = 1.2$, 2.1, 1H), 6.04 (d, $J = 2.4$ Hz, 1H), 3.27 (t, $J = 7.2$ Hz, 2H), 2.77 (t, $J = 7.6$ Hz, 2H); ^{31}P NMR δ-4; ESMS *m/z* 355 (M + H); and melting point = 170–171 °C.

4′-Phosphophloretin (4′-PP) was synthesized from 2,6-dihydroxy-4-phospho benzene and 4-hydroxy phenyl propionyl nitrile [18]. The 4′-phosphoester was resolved from the 2′-phosphoester by chromatography on silica gel using hexanes: dichloromethane: ethyl acetate (50:25:25). 2,6-Dihydro-4-phospho benzene was synthesized from phloroglucinol and dibenzyl phosphite in acetonitrile and triethylamine [19]. Prior to reaction with dibenzyl phosphite, phloroglucinol was dried at 105 °C under vacuum for 7 days. 2,6-Dihydro-4-phospho benzene was isolated by column chromatography on Dowex 1 using 25% methanol to elute the column. 4′-Phosphophloretin was purified by silica gel column chromatography developed with hexanes: dichloromethane: ethyl acetate (60:25:15). 4′-Phosphophloretin was analyzed by NMR and mass spectrometry. ^{1}H NMR (750 Hz, d_6 DMSO) δ 13.5 (s, 1H), 9 (br. s, 1H), 7.08 (d, $J = 8.2$ Hz, 2H), 7.06 (d, $J = 8.2$ Hz, 2H), 6.74 (s, 2H), 6.65 (d, $J = 8.2$ Hz, 2H), 6.62 (d, $J = 8.2$ Hz, 2H), 2.7 (t, $J = 7.5$ Hz, 5.1 Hz, 2H), 1.22 (s, 2H); ^{31}P NMR δ-4.8; ESMS *m/z* 355 (M + H); and melting point 178–179 °C.

4-Phosphophloretin (4-PP) was synthesized from 3-(4-dibenzyl phosphophenyl) propionyl chloride and phloroglucinol by Friedel-Crafts acylation in DMSO with anhydrous $AlCl_3$ [9,18]. The carboxylic acid of 3-(4-hydroxy)-cinnamic acid (5 g) was reacted with benzyl bromide in HMPT (hexamethylphosphoric triamide) for 1 h at 23 °C. The benzoate was collected and recrystallized from ethanol. The benzoate (5.04 g, 20 mmol) was added to 50 ml *n,n*-dimethylacetamide and cooled to 4 °C with stirring, and NaH was added (0.64 g, 25 mmol). The mixture was brought to 23 °C and 10 ml CCl_4 was added. Dibenzyl phosphite (5.6 g, 25.8 mmoles) in 25 ml *n,n*-dimethylacetamide was added and stirring was continued for 1 h at 23 °C. The reactants were diluted with 0.2 M acetate buffer, pH 4 (200 ml) and the di-benzyl phosphate ester was partitioned between water:hexane:ethyl acetate (50:25:25). The di-benzyl phosphate ester was reduced in volume, purified by chromatography on a silica gel column eluted with a 25–50% ethyl acetate gradient in hexanes, and dried at 75 °C under vacuum. The benzyl protecting groups were cleaved by catalytic hydrogenation with H_2 gas in ethyl acetate (100 ml) and 200 mg Pd/C for 24 h. 4-PP was purified as previously described [9]. 3-(4-phosphophenyl) propionyl chloride was synthesized from 3-(4-hydroxy) cinnamic acid and dibenzyl phosphite [19]. ^{1}HNMR (400 Hz, d_6 DMSO) δ 10.5 (br. s, 1H), 9.2 (br. s, 2H), 7.02 (d, 2H, $J = 8.2$ Hz), 6.8 (d, 2H, $J = 8.2$ Hz), 6.64 (d, 2H, $J = 8.4$ Hz), 6.6 (dd, $J = 2.5$, 1.5 Hz, 1H), 6.04 (d, $J = 2.5$ Hz, 1H), 3.3 (t, $J = 7.2$ Hz, 2H), 2.7 (t, $J = 7.5$ Hz); ^{31}P NMR δ-4.8: ESMS *m/z* 355 (M + H); and melting point 182 °C.

Phosphorylated phloretin derivatives were analyzed by thin layer chromatography using silica gel and methanol:H_2O (1:3) as the developing solvent. Spots were identified by UV absorption, I_2 [20] and visualized for phosphate esters using Hanes reagent [21]. Phosphophloretin derivatives were single spots and judged to be 90–94% of the UV absorbing material.

Na^+-dependent brush border membrane vesicle uptakes. Na^+-gradient driven uptakes of phosphate, alanine, or glucose into intestinal brush border membrane vesicles were performed using a rapid mixing rapid filtering device as previously described [5,6,9–15]. Na^+-dependent phosphate uptake into brush border membrane vesicles was performed using 100 μM [^{32}P] phosphate, 100 mM mannitol, 10 mM Hepes/Tris, pH 7.5, 100 mM NaCl or 100 mM KCl (uptake buffers). Na^+-dependent glucose uptake was determined using 100 μM [^{3}H] glucose, 10 mM Hepes/Tris, pH 7.5, 100 mM mannitol, and 100 mM NaCl or 100 mM KCl. Na^+-dependent alanine uptake was determined using 100 μM [^{3}H] alanine, 100 mM mannitol, 10 mM Hepes/Tris, pH 7.5, and 100 mM NaCl or 100 mM KCl. Uptakes were performed at 23 °C using 100 μg of brush border membrane protein.

Experiments examining the effect of phosphophloretin derivatives on Na^+-dependent uptakes were performed as described above using 10 nM to 10 μM phosphophloretin dissolved in 10 mM KOH:borate, pH 6.5. Phosphophloretin was added to the uptake solution immediately prior to addition of protein. In some experiments the effect of external phosphate on phosphophloretin inhibition of Na^+-dependent phosphate uptake was examined. In these experiments, phosphate concentration was varied between 25 and 500 μM. The effect of phosphate concentration on phosphophloretin inhibition of Na^+-dependent [^{32}P]phosphate uptake into intestinal brush border membrane vesicles was analyzed using the non-linear regression program, Enzfitter, Elsevier, Biosoft, Cambridge, UK.

In some experiments the time course of phosphate uptake into human intestinal BBMV was examined. Uptake of phosphate into BBMV was determined between 3 s and 30 min at 23 °C. Na^+-dependent uptakes were defined as uptake in the presence of NaCl minus uptake in the presence of KCl. All uptakes were performed in triplicate and the results are expressed as means ± SE.

Measurement of BBM alkaline phosphatase activity. Intestinal BBM alkaline phosphatase activity was measured using 1 mM *p*-nitrophenylphosphate and 100 μg BBM protein as previously described [17]. In experiments examining the effect of phosphophloretin derivatives on alkaline phosphatase activity the indicated phosphophloretin derivative was varied between 100 nM and 100 μM.

Results

Effect of phosphophloretins on Na^+-dependent phosphate uptake

The time course of phosphate uptake into human intestinal BBMV is shown in Fig. 1. Phosphate uptakes into BBMV in the presence of NaCl (closed circles, solid line), in the presence of KCl (open squares, dashed line), and in the presence of NaCl and 100 nM 2′-PP (open circles, solid line) are shown. Fig. 1 shows a 7-fold overshoot for phosphate uptake over equilibrium phosphate uptake in the presence of NaCl. Addition of 100 nM 2′-PP resulted in a 75–80% decrease in phosphate uptake without affecting phosphate uptake at equilibrium. During the course of these studies, the phosphate overshoot of equilibrium phosphate accumulation varied between 5- and 12-fold (mean = 7.8-fold, $n = 5$).

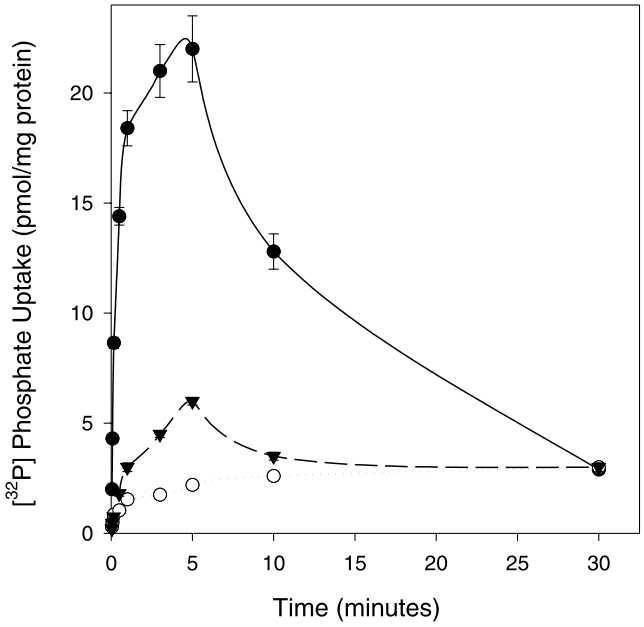


Fig. 1. Time course of phosphate uptake into human small intestinal BBMV. [^{32}P]phosphate uptake into human small intestine BBMV was determined as described in Materials and methods. Phosphate uptakes in the presence of NaCl (closed circles, solid line), in the presence of KCl (open circles, dotted line), and in the presence of NaCl + 100 nM of 2′-PP (inverse triangles, dashed line) were determined following 3 s to 30 min incubations at 23 °C. Results are means ± SE of triplicate determinations and representative of five experiments.

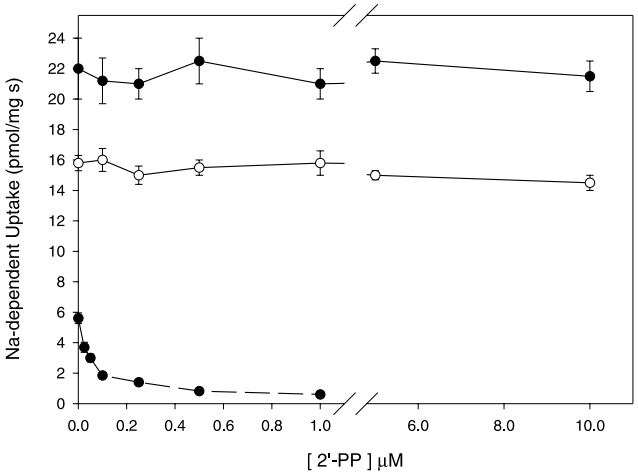
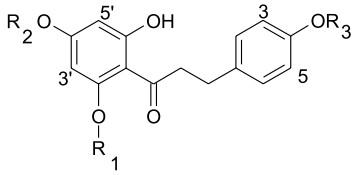


Fig. 2. Effect of 2′-PP on Na^+-dependent Cotransport Na^+ dependent [^{32}P]phosphate (solid circles, dashed line), Na^+-dependent [^{3}H] glucose (solid circles, solid line), or Na^+-dependent [^{3}H] alanine (open circles, solid line) uptakes were determined as described in Materials and methods. Results are means ± SE of triplicate determinations and representative of three separate experiments.

Fig. 2 shows the effect of 2′-phosphophloretin concentration on Na^+-dependent transport into BBM vesicles. 2′-PP inhibited Na^+-dependent phosphate uptake (solid circles, broken line) in a concentration-dependent manner with an apparent IC_{50} of 38 ± 6 nM ($n = 4$). Na^+-dependent glucose uptake (solid circles, solid line) and Na^+-dependent alanine uptake (open circles, solid line) were not affected by 2′-PP at concentrations 10 times that required for greater than 90% inhibition of Na^+-dependent phosphate uptake.

Studies examining the effect of phosphophloretins on Na^+-dependent phosphate uptake and alkaline phosphatase activity are summarized in Table 1. Na^+-dependent phosphate uptake was insensitive 4-PP and

Table 1
Effect of phosphorylated aromatics on Na-dependent phosphate uptake



Compound	Na^+-dependent phosphate uptake		Alkaline phosphatase activity
	IC_{50} (µM)	% Change	IC_{50} (mM)
2′-PP		Inhibition	
$R_2 = R_3 = H$	0.038 ± 0.006	92 ± 4	1.25 ± 0.25
$R_1 = HPO_4$			
4′-PP		Inhibition	
$R_1 = R_3 = H$	NM	15 + 4	0.96 ± 0.08
$R_2 = HPO_4$			
4-PP		Stimulation	
$R_1 = R_2 = H$	0.185 + 0.02	38 ± 12	0.350 ± 0.08
$R_3 = HPO_4$			
Phloretin	NM		0.692 ± 0.058

NM, not measurable. Results are means ± SE of triplicate determinations and three separate experiments. *p*-nitrophenyl phosphate concentration was 1 mM.

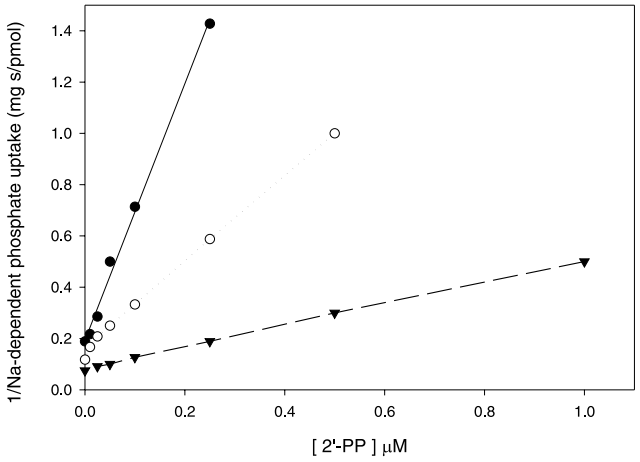


Fig. 3. Effect of [Phosphate] on 2′-PP Inhibition of Na^+-dependent phosphate uptake Na^+-dependent [^{32}P]phosphate uptake into intestinal brush border membrane vesicles was determined as described in Materials and methods. External phosphate concentration was 50 μM (open circles), 100 μM (closed circles), or 250 μM (solid triangles). 2′-PP concentration was varied between 10 nM and 1 μM. Results are means ± SE of triplicate determinations and representative of three experiments.

phloretin at concentrations below 100 μM. Addition of 4′-PP resulted in a 15% ± 4% ($n = 3$) inhibition of Na^+-dependent phosphate uptake at 500 nM 4′-PP. In contrast, Na^+-dependent phosphate uptake was inhibited more than 90% at 2′-PP concentrations above 100 nM. All of the phloretin derivatives examined were weak inhibitors of intestinal BBM alkaline phosphatase activity.

Effect of external phosphate concentration on 2′-PP inhibition of Na^+-dependent phosphate uptake

The effect of external phosphate concentration on 2′-PP inhibition of Na^+-dependent phosphate uptake is shown in Fig. 3. Fig. 3 is a Dixon plot of the effect of 50 μM phosphate (solid circles), 100 μM phosphate (open circles), and 250 μM phosphate (solid triangles) on 2′-PP inhibition of Na^+-dependent phosphate uptake. Increasing the external phosphate concentration decreased 2′-PP inhibition of Na^+-dependent phosphate uptake. The effect of phosphate concentration on 2′-PP inhibition of brush border Na^+-dependent phosphate uptake was analyzed by the method of Cornish-Bowden [22] at 50, 100, and 250 μM of 2′-PP. The intercept of the three straight lines was above the *X*-axis and to the right of the *Y*-axis, which is consistent with mixed inhibition by 2′-PP [23].

Discussion

2′-PP inhibition of Na^+-dependent phosphate uptake was measured in proximal small intestine brush border membrane vesicles isolated from human small intestine. The apparent IC_{50} for 2′-PP inhibition of Na^+-dependent phosphate uptake was 38 ± 8 nM (Fig. 2). The apparent IC_{50} for 2′-PP inhibition of human intestinal BBMV Na^+-dependent phosphate uptake was similar to that reported for rabbit intestinal brush border membrane vesicles and rat intestinal brush border membrane vesicles [11].

The effect of 2′-PP was specific for the Na^+/phosphate cotransporter and specific for 2′-PP. Na^+-independent phosphate uptake, Na^+-dependent glucose uptake, and Na^+-dependent alanine uptake were not affected by 2′-PP addition to the uptake media (Fig. 2). 4′-PP and 4-PP did not alter Na^+-dependent phosphate uptake into human intestinal brush border membrane vesicles, indicating that the effect of phosphophloretin on the Na^+/phosphate cotransporter was specific for the 2′-isomer (Table 1).

Table 1 indicates that BBMV esterase activity did not contribute to phosphophloretin inhibition of Na^+- dependent phosphate uptake. Although the phosphophloretin derivatives were inhibitors of intestinal BBMV alkaline phosphatase activity, the order of inhibitor potency for phosphophloretin inhibition of alkaline phosphatase was different from the order of inhibitor potency for phosphophloretin inhibition of Na^+- dependent phosphate uptake. Phosphophloretin inhibition of alkaline phosphatase hydrolysis of p-nitrophenyl phosphate followed the sequence: 4-PP > 4′-PP > 2′-PP > phloretin (Table 1). Phosphophloretin inhibition of Na^+-dependent phosphate uptake followed the sequence: 2′-PP ≫ 4′-PP > phloretin > 4-PP (Table 1). The effect of phloretin on human proximal small intestine brush border membrane Na^+-dependent phosphate uptake was similar to the effect of phloretin on Na^+-dependent phosphate uptake into K562 cells and human erythrocytes [24], and into rabbit proximal small intestine brush border membrane vesicles [9].

The effect of external phosphate on 2′-PP inhibition of Na^+-dependent phosphate uptake appeared to be competitive. Increasing external phosphate decreased 2′-PP inhibition of Na^+-dependent phosphate uptake (Fig. 3). Further examination of the results in Fig. 3 by a plot of the slope from Fig. 3 versus the reciprocal of the phosphate concentration and the method of Cornish–Bowden [22,23] indicated mixed inhibition (V_{max} and K_M inhibited).

The effect of 2′-PP on the V_{max} for Na^+-dependent phosphate uptake may be due to the off rate of 2′-PP from the Na^+-loaded cotransporter. If the 2′-PP off rate is much slower than the 3 seconds used for measurements of Na^+-dependent phosphate uptake, the cotransporter: Na^+:2′-PP complex would effectively be a dead-end complex. The resultant removal of a significant percentage of the cotransporter as a dead-end complex would result in a decrease in the apparent transport velocity and decreased V_{max}.

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