NaPO₄ cotransport type III (PiT1) expression in human embryonic kidney cells and regulation by PTH

Isabelle Fernandes, Richard Béliveau, Gérard Friedlander, and Caroline Silve. NaPO₄ cotransport type III (PiT1) expression in human embryonic kidney cells and regulation by PTH. Am. J. Physiol. 277 (Renal Physiol. 46): F543–F551, 1999.—The aim of the present study was to characterize the type(s) of NaPO₄ cotransporter expressed in the human renal cell line HEK-293 and its regulation by parathyroid hormone (PTH) in wild-type and cells transfected by the PTH/PTH-related protein (PTHrP) receptor. The results showed that human embryonic kidney HEK-293 cells expressed NaPO₄ cotransporter type III (PiT1) mRNA and protein. In contrast, type I (NPT1) or II (NPT2) cotransporter mRNAs were not expressed. Na⁺/dependent phosphate uptake followed a Michaelis-Menten model (apparent maximal transport rate and affinity constant: 23.32 ± 6.09 nmol PO₄/mg protein min⁻¹ and 0.147 ± 0.014 mM KH₂PO₄, respectively), was stimulated by phosphate deprivation (maximal increase 24.5 ± 0.8%, P < 0.001, after 15 h of phosphate deprivation), and was inhibited by increasing pH (3.6 ± 0.2-fold decrease at pH 8.5, P < 0.0001). It was inhibited in a time- and concentration-dependent fashion by PTH in HEK-293 cells stably transfected by PTHrP receptors but not in parental HEK-293 cells. Maximal inhibition of Na⁺/dependent phosphate transport was observed at 30 min after the addition of 72 nM PTH (1–34) (31.5 ± 2.4% inhibition, P < 0.01). PTH inhibition of phosphate transport was maintained in phosphate-deprived cells and reversed by both GF109203X (10⁻⁶ M) or staurosporine (5.5 nM), two protein kinase C inhibitors. Na⁺/dependent phosphate uptake was also significantly inhibited by phorbol 12-myristate 13-acetate (20.9 ± 3.9% inhibition, P < 0.001) but not by dibutyryl-cAMP (10⁻⁴ M) or forskolin (50 µM). The physiological role played by type III NaPO₄ cotransporter expression in the overall renal regulation of phosphate homeostasis remains to be established.

Human embryonic kidney HEK-293 cells

Three types of nonhomologous NaPO₄ cotransporters have recently been characterized at the molecular level (2, 31, 37, 39). Two of these types (types I and II) share 20% homology in amino acid sequences and are expressed almost exclusively in the renal proximal tubules (9, 10). Type I NaPO₄ transporter (designated NPT1, Npt1, and NaPi-1, respectively, in humans, mouse, and rabbit) (39) is expressed in the liver in addition to the kidney (14, 28). Its expression and activity are not regulated by phosphate deprivation or parathyroid hormone (PTH) status, and its role in the regulation of phosphate homeostasis remains unclear (2, 24, 31). Type II cotransporter (designated NaPi-2, NaPi-3 or NPT2, NaPi-4, NaPi-5, NaPi-6 or Npt2, and NaPi-7, respectively, in rat, human, opossum, flounder vessel, mouse, and rabbit) (2, 31, 37, 39) is a main target for regulation by dietary phosphate content and by PTH, and it ensures the bulk of tubular phosphate reabsorption (2, 24, 31). The key role of NPT2 in regulation of phosphate metabolism has recently been demonstrated by targeted inactivation of the Npt2 gene in mice (Npt2 –/– mice) (1) expressing a severe renal phosphate wasting. Type II cotransporter expression has been evidenced in brain (19), osteoclasts (16), lung (29), and very recently small intestine (NaPiIIb) (18) in addition to proximal tubules.

NaPO₄ cotransporters from the third type have been more recently identified and consist of proteins sharing low homology with those of the first two families. These proteins have been originally described as a family of cell surface receptors for gibbon ape leukemia virus (GALV) and murine amphotropic retrovirus (A-MuLV) (22, 23). In contrast to type I and type II NaPO₄ cotransports, type III NaPO₄ cotransports are ubiquitous in most species (21, 23, 30). The transcripts were found in relatively high abundance in total mRNA prepared from kidney (21, 23, 30) and were found to be expressed throughout the kidney by in situ hybridization (41). High levels of expression are also seen in tissues outside the kidney, including liver, lung, striated muscle, heart, and brain (22, 23). The GALV and A-MuLV receptors are coexpressed on most cells; nevertheless, each virus uses only its own receptor to enter cells (30, 32, 44, 45). These receptors are highly related phosphate symporters (23), and the human homologs are now designated Pit1 (GALV receptor, formerly GLVR1) and Pit2 (A-MuLV receptor, formerly GLVR2 or Ram1) (32, 44). Pit1 is 62% identical in amino acid sequence to Pit2 (22, 30). The Pit1 and Pit2 sequences share 20% identity with the mammalian type I and II NaPO₄ renal cotransporter sequences (22). The predicted amino acid sequences encoded by the Pit cDNAs are characteristic of integral membrane proteins, and hydrophy analysis suggests the presence of at least 10 potential transmembrane-spanning sequences, with a large hydrophilic domain near the center of each molecule (21, 22). This predicted topological profile differs from that of the renal transporters NPT1 and NPT2, which are modeled to contain between six and eight transmembrane domains (29). The central hydro-
philic domain of PiTs contains several consensus kinase C and consensus kinase A phosphorylation sites, which may be located intracellularly (21, 22). Thus, these type III NaPO4 cotransporters appear to represent a family of housekeeping NaPO4 cotransport systems. Little is known about their role in the overall phosphate homeostasis and on their cellular regulation. They share with type II cotransporter the ability to adapt to extracellular phosphate concentration in vitro, a feature not shared by type I transporters. It is interesting to note that brush border membrane (BBM) from Npt2−/− mice retains ~30% of the NaPO4 cotransport activity expressed in BBM from wild-type mice, suggesting that an NaPO4 cotransporter(s) other than Npt2 is active (1).

Although our knowledge on P1 homeostasis has been greatly enhanced since the molecular characterization of these three types of NaPO4 cotransporter systems, many aspects regarding the mechanisms involved in phosphate reabsorption by renal tubular cells remain unclear. Key information regarding the mechanisms involved in P1 uptake have been obtained by studies performed in cultured cells. However, most studies were performed before the molecular identification of the different NaPO4 cotransporter systems. Thus far, only one renal cell line (the opossum kidney OK cells) has been demonstrated to express the type II NaPO4 cotransporter and PTH/PTH-related protein (PTHrP) receptor, with a conserved PTH regulation of the transporter expression and activity (36). Other renal cell lines such as LLC-PK1 (3, 6) and Madin-Darby canine kidney (MDCK) (11) have also been used to characterize phosphate uptake, and, for both lines, regulation of NaPO4 cotransport activity by PTH after stable expression of the PTH/PTHrP receptor has been reported (5, 15, 17). However, the molecular characterization of the NaPO4 cotransporter type expressed in these cells has not been performed, and the physiological relevance of these studies needs to be documented. Conversely, the human embryonic kidney HEK-293 cell line has mostly been used as a tool to study the properties of transiently or stably transfected proteins, including NaPO4 cotransporter type II and PTH/PTHrP receptors (20, 35, 38, 42, 43). Compared with other renal cell lines that have also been used to study the properties of transfected proteins, such as MDCK and LLC-PK1, HEK-293 cells present the advantage of being of human origin rather than of canine (MDCK) or porcine (LLC-PK1) origin. Furthermore, we have recently demonstrated that PTH stimulates an increase in intracellular calcium in the absence of stimulation of inositol phosphates (InsPs) and cAMP production in wild-type HEK-293 cells but stimulates calcium release through an InsP pathway and induces cAMP production in cells expressing large numbers of PTH/PTHrP receptors after stable transfection of the receptor (20). The aim of the present study was to characterize the type(s) of NaPO4 cotransporter expressed in HEK-293 cells and its regulation by PTH in wild-type cells and in cells transfected by the PTH/PTHrP receptor.

### MATERIALS AND METHODS

#### Cell Culture

Wild-type HEK-293 cells were purchased from the American Type Culture Collection (Rockville, MD) and were used between passages 40 and 56. Parental HEK-293 cells (referred to as HEK/W) and HEK-293 cells stably transfected with the full-length human PTH/PTHrP receptor cDNA (referred to as HEK/T; see below) were maintained in a humidified atmosphere of 95% air-5% CO2 at 37°C in DMEM–Ham’s F-12 solution (GIBCO BRL, Gaithersburg, MD) supplemented with heat-inactivated FCS and newborn calf serum (5% each). 1 mM glutamine and 100 UI/ml penicillin G, and 50 µg/ml of streptomycin. For transport studies, cells were subcultured in 24-well plates coated with collagen I (100 µg/well; Sigma, St Louis, Mo); 2–5 × 10⁵ cells were seeded in each well in 0.5 ml of culture medium. Cells reached confluence in 4 days, at which time transport experiments were performed. Culture medium was replaced every other day.

#### Transfection

To obtain cells stably expressing the human PTH/PTHrP receptor, HEK/W cells were cotransfected with a pSV2 neo-plasmid and 3- to 20-fold excess of a full-length human PTH/PTHrP receptor cDNA inserted in pcDNA 1 plasmid as described (20). The clone selected for this study (HEK/T) stably expressed ~3.5 × 10⁵ receptors/cell and responded to PTH by an increase in cAMP and InsP productions and by a liberation of calcium from intracellular compartments via a phospholipase C-sensitive pathway (20).

#### Extraction of Cellular RNA

Total RNA was extracted from confluent cells grown in 56-cm² culture dishes according to the method of Chomczynski and Sacchi (7) and stored at −80°C. RNA concentration was determined by absorbance at 260 nm, and RNA integrity was evaluated by electrophoresis on 1% agarose gels containing ethidium bromide.

#### Reverse Transcription and Amplification by PCR of cDNA Coding for Different NaPO4 Cotransporter

cDNA was synthesize by reverse transcription of 5 µg of total RNA prepared from confluent HEK/W or HEK/T cells with the use of 400 U of Moloney murine leukemia virus RT (GIBCO BRL) and random hexamers as primers (1 µg of pDN6; Pharmacia Biotech, St. Quentin Yvelines, France) in a final volume of 50 µl with the use of 1 U of Taq DNA polymerase (GIBCO BRL) in the presence of 1.5 mM MgCl2, 0.25 mM each of deoxynucleotides (dNTP), and 0.5 pmol of each primer. The sequence of the oligonucleotide primers used for amplification for each NaPO4 cotransporter is described in Table 1 and was based on the human cDNA sequence for NPT1, NPT2, and Pit1 reported by Chong et al. (8), Magagnin et al. (29), and O’Hara et al. (32), respectively. Cycling parameters were as follows: 94°C/30 s, 55°C/30 s, and 72°C/30 s, for 35 cycles followed by 10 min at 72°C. Control PCR runs included amplification of samples 1) treated by deoxyribonuclease I (1 U/µg RNA; Pharmacia Biotech), 2) without reverse transcription, and 3) without template.

#### Restriction-Enzyme Analysis of PCR Product

The 215-bp PCR product obtained with the use of primers specific for Pit1 was digested by Msp I (GIBCO BRL) under conditions suggested by the manufacturer, and digestion...
products were visualized by ultraviolet illumination after electrophoresis on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide. Digestion of the PCR product generated two fragments (185 and 30 bp).

Crude Membrane Extraction and Western Blotting

Confluent HEK/W and HEK/T cells in 60-cm² plates were first washed twice with DMEM-Ham's F-12 and lysed with 3 ml of buffer A (5 mM Tris·HCl, pH 7.4, and 1 mM EDTA) containing 0.2 mM phenylmethylsulfonyl fluoride (a protease inhibitor) (Sigma, St. Quentin Fallavier, France). Then the plates were scraped with a disposable cell scraper, and lysates were removed and homogenized in a Dounce homogenizer (piston B) by 15 up-down strokes and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was recovered and centrifuged at 20,000 g for 30 min at 4°C. The pellet of crude membranes was solubilized in 30 μl of buffer A and preserved at −80°C.

SDS-PAGE was carried out according to the procedure of Laemmli (26). A mass of 20 μg of crude membranes was solubilized in 95 mM Tris·HCl (pH 6.8), 20% glycerol, 2% SDS, and 143 mM β-mercaptoethanol in a total volume of 20 μl. Proteins were separated by SDS-PAGE (10% gel), and gel was electrotransferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Blots were blocked with buffer B (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) for 1 h and then incubated overnight at 4°C with rabbit anti-PIT1 antisera and mouse anti-β-actin (Sigma) monodonal antibody, both at a dilution of 1:5,000 in buffer B. Polyclonal antibodies, raised against a portion of human PIT1 NaPO₄ cotransporter ranging from amino acids 408 to 421, were previously characterized (4). Blots were washed three times for 15 min with buffer B. To visualize primary antibodies bound, goat anti-rabbit IgG horseradish peroxidase conjugates and sheep anti-mouse IgG horseradish peroxidase conjugates (Bio-Rad) were added for an additional 1 h at a dilution of 1:5,000 in buffer B. Blots were then washed three times with buffer B. The antibody complexes were visualized with the use of an enhanced luminosol-based reagent [enhanced chemiluminescence (ECL) reagent; Amersham, Les Ullis, France] according to the manufacturer’s instructions. Exposure to ECL film (Amersham) varied from 15 s to 5 min.

Agonist and Antagonist Treatment of the Cells

Cultured medium was replaced 3 h before uptake by a DMEM-Ham's F-12 medium without serum, and agonist and antagonist treatment of the cells was performed as described (11, 12) in DMEM-Ham's F-12 with 0.1% BSA for the appropriate time period in an atmosphere of 5% CO₂-95% air at 37°C before uptake. Bovine PTH (bPTH)-(1–34) was dissolved in 10 mM acetic acid and 0.1% BSA, dibutyryl-cAMP (db-cAMP) in H₂O, and forskolin in ethanol. Phorbol 12-myristate 13-acetate (PMA), GF109203X, and staurosporine were dissolved in DMSO. Control cells were treated with a corresponding amount of vehicle; final DMSO and ethanol concentrations were <1/1,000. The concentrations of the stock solutions were as follows: PTH, 0.24 × 10⁻⁷ M; db-cAMP, 0.1 M; forskolin, 10 mg/ml; PMA, 1 mM; and GF109203X and staurosporine, 10⁻³ M. BSA, PTH, db-cAMP, forskolin, and PMA were from Sigma, and GF109203X and staurosporine were from Biomol (Plymouth, England, UK).

Phosphate Deprivation

Culture medium from subconfluent cells in 24-well plates was replaced by DMEM containing either no phosphate (Pi-depleted DMEM; GIBCO BRL) or P_i-depleted DMEM supplemented with 1.2 mM KH₂PO₄ (final concentration) and 10% FCS for 1–15 h before phosphate uptake measurements.

Phosphate Uptake Measurements

Transport of phosphate was measured as previously described (11, 12) with the use of 0.5 μCi/ml of [³²P]KH₂PO₄ (Amersham) and 0.1 mM KH₂PO₄, except that Na⁺-independent phosphate uptake was measured in the presence of 137 mM N-methylglucamine-hydrochloride and not choline chloride. Na⁺-dependent Pi transport (nmol·mg protein⁻¹·10 min⁻¹) is the difference between total Pi transport and Na⁺-independent Pi transport. [³²P]Phosphate uptake without Na⁺ was <7% of that in the presence of Na⁺ and did not vary with different experimental protocols at the concentration used in these studies, including treatment with PTH, db-cAMP, PMA, GF109203X, and staurosporine. To study the pH effect, the uptake media were equilibrated at the indicated pH (see results).

Alanine Uptake Measurements

Na⁺-dependent transport of alanine was measured with the use of 5 μCi/ml of L-[³H]alanine (Amersham), as previously described (11).

Kinetics of Na⁺-Dependent Phosphate and Alanine Transports

The uptake of phosphate was measured for 10 min in transport media (pH 7.4) containing different phosphate concentrations (12.5, 25, 50, 100, 250, 500, and 1,000 μM) at 37°C. The uptake of alanine was measured for 5 min in the media transport containing different alanine concentrations (0.1, 0.25, 0.5, 1, 2.5, and 5 mM) at 37°C. Apparent affinity constant (Kₘ) and maximal transport rate (Vₘ₅ₓ) were calculated by nonlinear curve fitting, assuming Michaelis-Menten kinetics.

Expression of Data and Statistical Analysis

Unless otherwise stated, results are expressed as ± SE. All transport experiments were performed in triplicate. For statistical analyses performed, see figure legends. P < 0.01 was considered significant. Similar results were obtained in sham-transfected and wild-type HEK-293 cells.

RESULTS

Identification of NaPO₄ Cotransporters Expressed in HEK Cells by RT-PCR

When RT-PCR was performed from RNA prepared from HEK/T and HEK/W cells with the use of oligo-

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Table 1. Sequence and position of primers used to amplify different NaPO₄ cotransporters

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size of Amplified Product</th>
</tr>
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<tbody>
<tr>
<td>NPT1</td>
<td>5′ AAC GAG GCC GAC TTA CTT CTA TGA 3′</td>
<td>237 bp</td>
</tr>
<tr>
<td></td>
<td>5′ GCT GGA CCA GGG AGG ATG TGA GGT 3′</td>
<td>302 bp</td>
</tr>
<tr>
<td>NPT2</td>
<td>5′ GTC ATC CAG AAG GTC ATC A3′</td>
<td>215 bp</td>
</tr>
<tr>
<td>PIT1</td>
<td>5′ TAC AAT TCG ACT CAA GGG CTA CGT 3′</td>
<td>302 bp</td>
</tr>
<tr>
<td></td>
<td>5′ GCC GAG ACG AAC CAA GAC ATC A3′</td>
<td>237 bp</td>
</tr>
</tbody>
</table>

Primers in bold letters are complementary to the (−) strand. NPT1, type I NaPO₄ cotransporter; NPT2, type II NaPO₄ cotransporter; PIT1, type III NaPO₄ cotransporter.
Characterization of Phosphate Uptake in HEK Cells

Time course. The time course of Pi uptake in the presence of NaCl or N-methyl-D-glucamine illustrates the Na⁺ requirement for Pi entry in HEK cells (Fig. 3). Phosphate uptake by HEK cells measured in medium containing Na⁺ increased linearly with incubation time for the first 20 min at 37°C and was maximal at ~30 min, at which time phosphate uptake was 26.9 ± 0.9 nmol Pi/mg protein (3 independent experiments) (Fig. 3). When Na⁺ was replaced with equimolar N-methyl-D-glucamine, phosphate uptake did not significantly change as a function of time and was ~7% of that measured in the presence of Na⁺ (0.4 ± 0.04 nmol Pi/mg protein). All other measurements of phosphate uptake were performed under linear uptake rates of 10 min.

Kinetic analyses. To determine the apparent kinetic parameters for phosphate, phosphate uptake was measured as a function of extracellular phosphate concentration. Na⁺-dependent phosphate uptake was a saturable process and followed a Michaelis-Menten model. Analysis of the kinetics parameters, calculated by nonlinear curve fitting, indicated similar values for apparent V_{max} and K_{m} in HEK/W and HEK/T cells (Fig. 4).

Dependence on extracellular pH. Na⁺-dependent phosphate uptake was markedly decreased by raising external pH from 7.4 to 8 and further declined at pH 8.5 (2.3 ± 0.4- and 3.6 ± 0.2-fold decreases, respectively, at pH 8 and pH 8.5 compared with the uptake measured at pH 7.4; P < 0.0001, n = 7; Fig. 5). Increasing external pH from 7.4 to 8.5 had no significant effect on Na⁺-independent phosphate uptake (Fig. 5). Similar results were obtained in HEK/W and HEK/T cells (results are shown for HEK/W).

Modulation by phosphate deprivation. Incubation of HEK cells in phosphate-deprived culture medium increased phosphate uptake in a time-dependent manner.
compared with that in cells incubated with medium containing 1.2 mM phosphate for the same time (Fig. 6). Phosphate uptake significantly increased 1 h after phosphate deprivation with a maximum uptake at 15 h after phosphate deprivation (14.6 ± 0.2 and 24.5 ± 0.8% increase, respectively, compared with that measured in phosphate-containing medium; \( P < 0.01 \) and \( P < 0.001 \), \( n = 3 \)). Na\(^+\)-independent phosphate uptake was not significantly changed by phosphate deprivation of the culture medium (data not shown). Similar results were obtained in HEK/W and HEK/T cells (results are shown for HEK/W).

**PTH Regulation of Phosphate Transport in HEK Cells**

PTH inhibited Na\(^+\)-dependent phosphate uptake in a time- and concentration-dependent fashion in HEK/T cells. PTH inhibition of phosphate uptake was rapid and transient. Maximal inhibition of Na\(^+\)-dependent phosphate was observed at 30 min after the addition of 72 nM bPTH-(1—34) (31.5 ± 2.4% inhibition, \( P < 0.01 \)), after which time Na\(^+\)-dependent phosphate uptake increased progressively to reach values not significantly different from those observed in control cells after 1 h (Fig. 7). PTH inhibition of phosphate uptake was maintained in phosphate-deprived cells; the time
course of the inhibition was similar to that seen in non-phosphate-deprived cells (Fig. 7). The PTH inhibitory effect, when studied under these conditions, is higher in absolute terms in P_i-adapted cells compared with non-P_i-adapted cells (5.2 ± 0.25 and 4.0 ± 0.31 nM PTH for each curve). As depicted in Fig. 8, PTH exerted its inhibition on Na^+-dependent phosphate uptake in a concentration-dependent manner, and Na^+-dependent phosphate uptake was significantly inhibited by 7.2 nM PTH (18.6 ± 3.5% inhibition; P < 0.01), maximal inhibition was obtained by 240 nM PTH (Fig. 8). PTH inhibition of phosphate uptake was related to a decrease in V_{max} of the transporter PTH (21.3 ± 0.9% inhibition; P < 0.001, n = 4; Fig. 9). This effect of PTH was specific for phosphate transport, as PTH had no effect on Na^+-dependent alanine transport (Fig. 10). PTH had no effect on phosphate uptake in HEK/W cells (Fig. 8).

Pathways Involved in PTH-Induced Inhibition of Phosphate Uptake

In an attempt to characterize the cellular pathways involved in PTH-induced inhibition of Na^+-dependent phosphate uptake, the effects of exogenous db-cAMP, forskolin, and PMA on Na^+-dependent phosphate uptake were first characterized. Previous studies from our group (20) and others (35, 38) have demonstrated that PTH stimulates cAMP and InsP production in HEK/T and T-24 cells. Results showed that neither db-cAMP (10^{-6} M) nor forskolin (50 μM) inhibited Na^+-dependent phosphate uptake (Fig. 11). The effects of PTH in the presence of GF109203X (10^{-6} M) or staurosporine (5.5 nM) [two inhibitors of protein kinase C (PKC)] were then studied. PTH inhibition of phosphate transport was reversed by both PKC inhibitors (Fig. 11). Neither compound alone had a significant effect on Na^+-dependent phosphate uptake. PTH effect was studied in two different clones of PTH/PTHrP receptor-transfected cells (expressing approximately the same number of receptors), and similar results were obtained in both clones.

Fig. 8. Effect of increasing concentrations of bPTH-(1—34) on Na^+-dependent phosphate uptake by HEK/T (■) and HEK/W (▲) cells. Cells were incubated in presence of indicated PTH concentrations for 30 min, and Na^+-dependent phosphate uptake was measured as described in MATERIALS AND METHODS. Results are means ± SE of 3 experiments performed in triplicate and are expressed as percentage of values measured in absence of PTH. For each curve, statistical analysis was performed with the use of 1-way ANOVA (P < 0.001 and P > 0.05 for HEK/T and HEK/W cells, respectively). Values in presence of PTH were compared with those in its absence by Newman-Keuls test. *P < 0.01 compared with value in absence of PTH for each curve.

Fig. 9. Effect of PTH on kinetics of Na^+-dependent phosphate uptake by HEK/T cells. Cells were incubated for 30 min in absence (●) or presence (▲) of 72 nM bPTH-(1—34), and Na^+-dependent phosphate uptake as a function of extracellular phosphate concentrations was measured as described in MATERIALS AND METHODS. Inset: kinetic parameters of Na^+-dependent phosphate uptake calculated by nonlinear curve fitting, assuming Michaelis-Menten kinetics. Data are means ± SE of 6 experiments performed in triplicate. K_{m} and V_{max} were compared with the use of Student’s t-test. **P < 0.001, comparison of V_{max} measured in cells incubated in absence with those incubated in presence of PTH.

Fig. 10. Effect of PTH on kinetics of Na^+-dependent alanine (ala) uptake by HEK/T cells. Cells were incubated for 30 min in absence (●) or presence (▲) of 72 nM bPTH-(1—34), and Na^+-dependent alanine uptake as a function of extracellular alanine concentrations was measured as described in MATERIALS AND METHODS. Inset: kinetic parameters of Na^+-dependent alanine uptake calculated by nonlinear curve fitting, assuming Michaelis-Menten kinetics. Na^+-dependent alanine uptake was calculated by subtracting uptake measured under Na^-free conditions from that observed in presence of Na^+ at each alanine concentration. Data are means ± SE of 3 experiments performed in triplicate. PTH had no significant effect on alanine uptake (P > 0.05).


**DISCUSSION**

The results of this study showed that wild-type and HEK/T cells expressed NaPO₄ cotransporter type III (PTT1) but not type I (NPT1) or II (NPT2). Na⁺-dependent phosphate uptake was stimulated by phosphate deprivation and inhibited by increasing pH. It was inhibited in a time- and concentration-dependent fashion by PTH in HEK/T but not in HEK/W cells. This effect of PTH persisted in phosphate-deprived cells and appeared to be mediated via PKC activation.

That HEK-293 cells express NaPO₄ cotransporter type III (PTT1) and not type I or II is indicated by the following results. 1) A PCR product corresponding to the Pit1 cDNA sequence was amplified with the use of primer specific for the human Pit1 sequence, whereas no PCR products were obtained with primers specific for the NaPO₄ cotransporter types I and II. 2) Western blots using an antibody directed against an epitope present in the Pit1 protein allowed for the detection of a protein of the expected size. 3) The study of the regulation of the NaPO₄ cotransport activity by extracellular pH demonstrated that an increase in pH led to an important decrease in transport activity, a previously demonstrated property of NaPO₄ cotransport type III. In contrast, it is known that the activity of NaPO₄ cotransport type II is increased by raising extracellular pH (2, 29), whereas that of cotransport type I is not modified (2). A slight decrease in type IIb-associated NaPO₄ cotransport activity was recently described (18). However, this pH dependency was small, and, furthermore, NaPO₄ cotransporter type IIb is not expressed in the kidney. These results strongly suggest that HEK-293 cells do not express type I or II NaPO₄ cotransporter, although it cannot be ruled out that they express an as-yet-unidentified NaPO₄ cotransporter. The absence of NaPO₄ cotransporter type I has recently been reported in HEK-293 cells (42), in agreement with our data. In the same study, NaPO₄ cotransport type II expression was reported. However, an important decrease in “native” NaPO₄ cotransport activity was observed as a function of an increase in extracellular pH, which is difficult to reconcile with the pH dependency described for type II cotransport activity. The observed pH dependency of NaPO₄ cotransport activity would be compatible with the presence of NaPO₄ cotransport type III, although the molecular expression of NaPO₄ cotransport type III was not assessed.

Having characterized the type of NaPO₄ cotransport expressed in HEK-293 cells, and, in an effort to shed some light on and integrate various data from the literature describing regulation of NaPO₄ cotransport activity by PTH, we next studied the regulation of NaPO₄ activity by PTH in HEK/W cells and in HEK/T cells. A previous study from our laboratory (20) demonstrated that PTH induces an increase in intracellular calcium concentration in the absence of stimulation of InsP and cAMP production in HEK/W cells but stimulates calcium release associated with an increase in InsP pathway production (thus most likely stimulating PKC activation) and induces cAMP production in HEK/T cells. In the present study, it is demonstrated that PTH stimulation of HEK/W cells does not regulate NaPO₄ cotransport activity. In contrast, PTH stimulation of HEK/T cells leads to a time- and concentration-dependent inhibition of NaPO₄ cotransport activity associated with a decrease in the Vₘₐₓ of the cotransport, without change in affinity for phosphate. This effect of PTH was specific for phosphate, as alanine uptake was not modified by PTH treatment of the cells. The results obtained in HEK/W and HEK/T cells are compatible with the possibility that PTH inhibits NaPO₄ cotransport activity in HEK/T cells via PKC-activated pathways. This hypothesis is compatible with the presence of potential phosphorylation sites by PKC in the cotransporter type III sequence (21, 32). In support of that possibility, NaPO₄ cotransport was also inhibited by PKC activation, but not that of protein kinase A (PKA), and the addition of two PKC inhibitors reversed PTH inhibition. Because HEK-293 cells express Na-P₇ type III (PTT1) and not Na-P₇ type I and II, it seems reasonable to assume that the NaPO₄ cotransport activity shown in the present study to be inhibited by PTH and PKC activation was mediated by Na-P₇, type III. Recently, it has been shown that PTH inhibition of Na-P₇ type II cotransport activity is associated with the stimulation of Na-P₇ type II transporter endocytosis, followed by degradation (34). It is unlikely that PTH inhibition of Na-P₇ type III cotransport activity in HEK/T cells involves endocytosis, because 1) the effect of PTH in HEK/T cells is transient, and 2) Na-P₇ cotransport types II and III are expressed at the BBM and basolateral membrane, respectively, of cells (unpublished results). These membranes have different structural properties, suggesting that the interaction between Na-P₇ cotransport types II and III and the cytoskeleton is different.

In a recent study by Lederer et al. (27), it was demonstrated that PTH stimulation of OK cells leads to...
a decrease of Na-P cotransport activity associated with a decrease of type II expression at the membrane via PKA activation, whereas PTH activation of PKC induced a decrease in Na-P cotransport activity without a change in expression in type II. It has been demonstrated that OK cells express type III cotransport in addition to that of type II (4). Because the activity of these transporters cannot be readily differentiated, it is possible that part of the PTH effect described resulted from PTH regulation of both type III and type II cotransport activities. An absence of regulation by PTH of Na-P type III cotransporter protein (studied by Western blot) and activity has been reported in OK cells (4) and in mouse distal convoluted tubule (MDCT) cells (40), respectively. However, in these studies, the PTH effect was studied after 2 h (OK cells) and 4 h (MDCT cells) of incubation with PTH, at which time we did not observe a PTH inhibitory effect.

We found that PTH inhibits NaPO₄ cotransport activity in HEK-293 cells, in contrast to the effect of PTH in two other cell lines, namely, MDCK and LLC-PK (5, 15, 17). When these latter cell lines were transfected by PTH/PTHrP receptors, it was found that PTH stimulates NaPO₄ activity. Although the NaPO₄ cotransporter expressed by these cell lines has not been characterized, it is tempting to speculate that it is NaPO₄ cotransporter type III. Similarly, it has been reported that PTH stimulates NaPO₄ cotransport activity in osteoblast-like cells that do not express type I or II NaPO₄ cotransporter but do express type III cotransporter (33). Thus these data would suggest that regulation of type III NaPO₄ cotransporter is cell specific. However, it is interesting to point out that in both cases (PTH inhibition or stimulation of NaPO₄ cotransport activity), the PTH effect appears to be mediated by an activation of PKC, not PKA. The regulation of NaPO₄ cotransport activity observed in HEK-293 cells is similar to that described in primary cultures of renal proximal tubular cells (13, 25). Interestingly, these cells at confluence do not express NaPO₄ cotransporter mRNA type II or I but do express type III (unpublished results), and NaPO₄ cotransport activity in these cells is rapidly inhibited by PKC activity (13).

In conclusion, the renal human cell line HEK-293 expresses only type III NaPO₄ cotransporter, but not type I or II. Regulation of this transporter activity by PTH in these HEK/T cells leads to an inhibition of the cotransporter that most likely involves PKC activation but not an increase in intracellular calcium or CAMP concentrations. These results underline the possibility that in cell lines expressing type II cotransporter, but also type III, such as OK cells, the observed PTH effect on NaPO₄ cotransport activity results from a regulation of different NaPO₄ cotransport activities. These findings may explain some discrepancies observed in studies describing the mechanisms involved in PTH regulation of NaPO₄ cotransporter activity in different cell lines, including subclones of OK cells. The physiological role played by type III NaPO₄ cotransport in the overall renal regulation of phosphate homeostasis remains to be established; keep in mind that 1) mRNA expression has been evidenced throughout the kidney (41), and 2) protein expression has been demonstrated at the basolateral and not apical membranes prepared from rat renal cortices (unpublished results). Because type III NaPO₄ cotransport is ubiquitously expressed and is located in the basolateral membrane of proximal tubules, it is unlikely that its main role is to regulate phosphate reabsorption by the renal proximal tubule. If the PTH regulation evidenced is taking place in vivo, which remains to be determined, one could speculate that it participates in the cellular phosphate metabolism.

We are grateful to Maurice Bichara for a careful reading of the manuscript.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, and the Association pour l’Utilisation du Rein Artificiel.

Isabelle Fernandez is a recipient of a fellowship from the Ministère de la Recherche et de la Technologie.

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Received 11 January 1999; accepted in final form 11 June 1999.

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