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# *p*-Aminohippuric Acid Transport at Renal Apical Membrane Mediated by Human Inorganic Phosphate Transporter NPT1

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**Organic anions are secreted into urine via organic anion transporters across the renal basolateral and apical membranes. However, no apical membrane transporter for organic anions such as *p*-aminohippuric acid (PAH) has yet been identified. In the present study, we showed that human NPT1, which is present in renal apical membrane, mediates the transport of PAH. The $K_m$ value for PAH uptake was 2.66 mM and the uptake was chloride ion sensitive. These results are compatible with those reported for the classical organic anion transport system at the renal apical membrane. PAH transport was inhibited by various anionic compounds. Human NPT1 also accepted uric acid, benzylpenicillin, faropenem, and estradiol-17β-glucuronide as substrates. Considering its chloride ion sensitivity, Npt1 is expected to function for secretion of PAH from renal proximal tubular cells. This is the first molecular demonstration of an organic anion transport function for PAH at the renal apical membrane.** 

Renal secretion of organic anions in renal proximal tubules is of great interest, since most organic anions, including endogenous compounds and xenobiotics, are removed from the body through active secretion via organic anion transport systems across renal epithelial cells (1–3). On the basolateral membrane, the organic anion transporter OAT1 mediates the active transport of *p*-aminohippuric acid (PAH; a typical substrate of organic anion transport systems) and the accumulation of organic anions in the epithelial cells (4–6). Organic anions are also transported at the renal apical membrane (7, 8), although the molecular nature of the transporter for PAH on the apical membrane has not yet been determined.

Recently, we cloned and functionally characterized human type I sodium-dependent inorganic phosphate (Pi) transporter NPT1 (9). NPT1 is localized in the apical membrane of renal proximal tubules (10), and takes up phosphate from urine to maintain the phosphate ion level in the blood. Based on hybrid depletion experiments, the type I Na/Pi transporter was presumed to make little contribution to phosphate reabsorption (11), suggesting that it may have another role in the kidney.

Rabbit NaPi-1 and mouse Npt1 transport anionic compounds, including β-lactam antibiotics (12–15), but NPT1-mediated transport of organic anions has not been fully characterized yet. In the present study, we further examined the hypothesis that NPT1 is a physiologically and pharmacologically important organic anion transporter at the renal apical membrane by measuring the transport of PAH by HEK293 cells transfected with human NPT1.

## MATERIALS AND METHODS

*Materials.* *p*-[glycyl-$^3$H]Aminohippurate (2.5 Ci/mmol), [$^3$H]estradiol-17β-glucuronide (55 Ci/mmol) and [$^{14}$C]indomethacin (22.3 mCi/mmol) were purchased from New England Nuclear (Boston, MA). [$^{14}$C]Benzylpenicillin (56 mCi/mmol) was purchased from Amersham International, Ltd. (Buckinghamshire, England). [$^{14}$C]Uric acid (50 mCi/mmol) was obtained from ARC Inc. (St. Louis, MO). [$^{14}$C]Faropenem (52 mCi/mmol) and unlabeled faropenem were kindly provided by Suntory Co. Ltd. (Tokyo, Japan). 2-Ketoglutaric acid and probenecid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest purity available. HEK293 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan).

*Human NPT1 cDNA cloning using RT-PCR.* The cDNA of human NPT1 was isolated from human kidney poly(A)$^+$ RNA (Clontech, Palo Alto, CA) using RT-PCR. The specific primers of human NPT1

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were 5′-CTTCAGCCGCATATGCAAATGCATAACCGG-3′ and 5′-CAGGCTCTGGAATTCCTCTGTTCACACTT-3′. The PCR product was digested by *Nde*I and *Eco*RI, and was subcloned into pSK$^{-}$ vector. Sequence analysis showed that this clone was identical to the human NPT1 cDNA reported previously (16).

*HEK293 expression system.* The full-length human NPT1 cDNA was subcloned into the expression vector pCAGGS followed by the insertion of a FLAG sequence at the N-terminal of NPT1. The construct pCAGGS/hNPT1 was used to transfect HEK293 cells by means of the calcium phosphate precipitation method as described previously (17). The cells were cultivated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Life Technologies, Inc., Tokyo, Japan), 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified incubator at 37°C under 5% $CO_2$ for 24 h, then transfected with pCAGGS/hNPT1 plasmid vector or pCAGGS plasmid vector alone.

Antiserum raised against a synthetic peptide consisting of the 15-amino-acid FLAG peptide coupled to KLH (keyhole limpet hemocyanin) was obtained from a rabbit after immunization. To confirm the expression of human NPT1 in HEK293 cells, the human NPT1-transfected cells were harvested and solubilized in RIPA buffer (10 mM Tris–Cl (pH 7.8)–1% NP-40–0.1% sodium deoxyglycholate–0.1% SDS–0.15 M NaCl–1 mM EDTA–10 $\mu$g/ml aprotinin–1 mM phenylmethylsulfonyl fluoride) containing 8 M urea. Western blot analysis was performed with the cell lysate. To identify the antibody specificity, the antibody was incubated with 1 $\mu$g of the immunogen peptide before use.

*Uptake measurement.* At 48 h after transfection, the cells were harvested and suspended in the transport buffer [125 mM Na gluconate, 4.8 mM K gluconate, 5.6 mM D-glucose, 1.2 mM Ca gluconate, 1.2 mM Mg gluconate, and 25 mM Hepes (pH 7.4)]. The cells and the transport buffer containing a radiolabeled test compound were incubated at 37°C separately for 10 min and mixed to initiate uptake. At appropriate times, 200-$\mu$l aliquots of the mixture were withdrawn, and the cells were separated from the medium by centrifugation through a layer of silicon oil and liquid paraffin mixture with a density of 1.03 g/ml. The cell pellets were solubilized in 3 N KOH, then neutralized with HCl, and the associated radioactivity was quantitated in a liquid scintillation counter (Aloka, Tokyo, Japan). Cellular protein content was determined according to the method of Bradford (18) using bovine serum albumin as the standard.

For efflux experiments, transfected HEK293 cells were washed in the standard reaction buffer (125 mM Na gluconate, 4.8 mM K gluconate, 5.6 mM D-glucose, 1.2 mM Ca gluconate, 1.2 mM Mg gluconate and 25 mM Hepes (pH 7.4), resuspended in the transport buffer containing 100 $\mu$M [$^3$H]PAH (2 $\mu$Ci) and incubated for 10 min. The cells were then pelleted by centrifugation at 10,000 rpm for 10 s and efflux was initiated by resuspending the cells in the transport buffer. The reaction was terminated by separating the cells from the medium by a centrifugal filtration technique as described previously (19).

*Statistical methods.* Results are given as means ± standard error of the means (SEM). Statistical analysis was performed with Student's *t* test. The criterion of statistical significance was deemed to be a *P* value of less than 0.05.

## RESULTS

The expression of NPT1 protein in NPT1-transfected HEK293 cells was confirmed by Western blot analysis. For this, we generated polyclonal antibody against the FLAG tag. Figure 1 shows that the anti-FLAG antiserum detected 80-kDa and 38-kDa bands from HEK293 cells transfected with human NPT1. Both the 80- and 38-kDa bands disappeared upon immunoabsorption of the anti-FLAG antibody with an excess of the FLAG peptide used for immunization. Human NPT1 cDNA coded a protein of 467 amino acid residues with a predicted nonglycosylated molecular mass of 51 kDa. This molecule presumably undergoes post-translational modifications such as glycosylation.

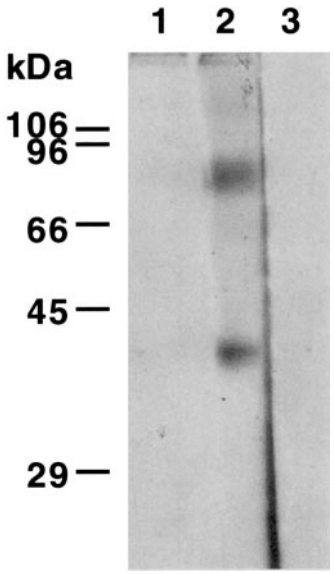


**FIG. 1.** Immunoblot analysis of the human NPT1. Electroblotted brush-border membrane proteins were assayed with an antiserum against FLAG peptide. Lanes 1 and 2 are results obtained in mock-transfected and FLAG-hNPT1-transfected cells, respectively. Lane 3 shows the result of immunoabsorption of FLAG-hNPT1 with FLAG-peptide. Molecular weight is shown on the left.

To characterize the organic anion transport via human NPT1, we examined the uptake of [$^3$H]PAH using the HEK293 cell expression system. [$^3$H]PAH transport by human NPT1 was observed in a chloride ion-free condition and attained a steady-state after 1 min (Fig. 2). To determine the driving force of the transport, the ion dependence on the NPT1-mediated PAH uptake was examined. The uptake of [$^3$H]PAH was independent of sodium ions (Fig. 3A), but was affected by chloride ions (Fig. 3B). When the concentration of chloride ions was increased, the transport rate of [$^3$H]PAH decreased. Moreover, the transport of [$^3$H]PAH was pH-dependent under a chloride ion-free condition (Fig. 3C). Thus, chloride ions and pH influenced the PAH transport via NPT1. [$^3$H]PAH uptake via NPT1 was saturable and followed Michaelis–Menten kinetics with a $K_m$ value of 2.66 ± 1.02 mM and a $V_{max}$ value of 940 ± 155 pmol/mg protein/30 s (mean ± SEM, $n$ = 3) (Fig. 4), indicating that NPT1-mediated transport of PAH shows relatively low affinity.

The substrate selectivity of human NPT1 was investigated by means of an inhibition experiment in which uptake of [$^3$H]PAH (100 $\mu$M) was measured in the presence of 5 mM anionic compounds, unless otherwise noted (Table 1). The [$^3$H]PAH uptake was strongly

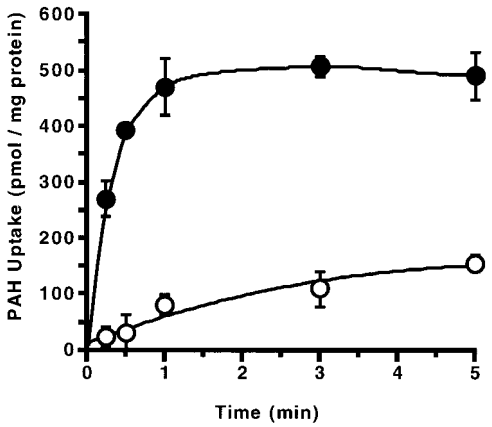


**FIG. 2.** Time course of [$^3$H]PAH uptake in NPT1-expressing HEK293 cells. At 48 h after transfection of HEK293 cells with hNPT1/pCAGGS, the cells were harvested for transport study using the silicon layer method. Uptake of [$^3$H]PAH (100 $\mu$M) was measured at the indicated time. The closed and open symbols represent results obtained in HEK293 cells transfected with NPT1/pCAGGS and pcAGGS vector alone, respectively. The results are shown as means ± SEM of three independent experiments.

inhibited by probenecid, $\beta$-lactam antibiotics (i.e., benzylpenicillin and faropenem), salicylate and indomethacin. The anion transport inhibitor DIDS also reduced [$^3$H]PAH uptake. Although 2-ketoglutaric acid or succinic acid was not inhibitory, it was difficult to detect the inhibitory effect of uric acid because of its solubility.

We measured the uptakes of several anions that were inhibitory toward PAH uptake by NPT1 to confirm that they are substrates of NPT1. As shown in Fig. 5, [$^{14}$C]uric acid, [$^{14}$C]benzylpenicillin, [$^{14}$C]faropenem and [$^3$H]estradiol-17$\beta$-glucuronide were transported into NPT1 cDNA-transfected cells. Although NPT1-induced [$^{14}$C]Indomethacin transport was relatively small compared to that by mock-transfected cells, the transported amount was significant. Taken together, these results suggested that NPT1 accepts various anionic compounds as substrates.

Since PAH uptake via NPT1 was sensitive to chloride ions, as shown in Fig. 3B, the direction of PAH transport via NPT1 was expected to be from the cell to the extracellular fluid *in vivo*. We tested the effect of chloride ions on efflux of [$^3$H]PAH in the cell-to-medium direction. At 2 min after initiation of efflux, the remaining amounts of preloaded [$^3$H]PAH were 44.4 ± 0.49% and 40.1 ± 0.36% in the presence or absence of chloride ions, respectively (Fig. 6). Therefore, [$^3$H]PAH efflux via human NPT1 is independent of extracellular chloride ions, so PAH was thought to be transported in the cell-to-extracellular direction. Moreover, extracellular PAH or phosphate ions did not affect [$^3$H]PAH efflux in NPT1-expressing cells (Fig. 6), though indomethacin inhibited [$^3$H]PAH efflux. This may be because of the rapid and extensive accumulation of indomethacin in the cells (Fig. 5), resulting in competition with efflux of [$^3$H]PAH. Moreover, extracellular pH did not affect [$^3$H]PAH efflux. At 2 min after initiation of efflux, the remaining amounts of preloaded [$^3$H]PAH were 42.8 ± 1.82% and 43.3 ± 2.15%, at pH 5.5 and 8.5, respectively ($n$ = 3). This result indicated that hydroxyl ion is not the driving force of the PAH transport by NPT1.

## DISCUSSION

It has been reported that the PAH excretion process at the renal membrane is voltage-sensitive and is possibly mediated by a PAH/anion exchanger or PAH/2-ketoglutarate exchanger (3), but the molecular nature of the transporter has not yet been clarified. In this study, we have demonstrated that human NPT1 transports PAH, uric acid and other organic anions, includ-

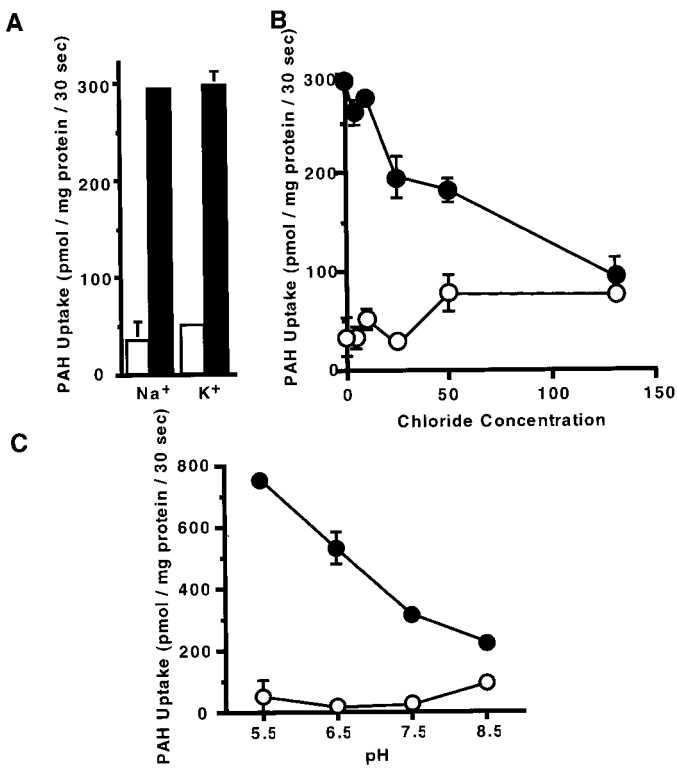


**FIG. 3.** Sodium (A), chloride (B), and pH (C) dependences of [$^3$H]PAH uptake by human NPT1 expressed in HEK293 cells. (A) Uptake of [$^3$H]PAH (100 $\mu$M) was measured in the presence or absence of sodium ions (labeled as "Na" and "K," respectively). In the sodium-ion free experiment, sodium ions were replaced with potassium ions. (B) Uptake of [$^3$H]PAH (100 $\mu$M) was measured in the medium containing various concentrations of chloride ions. Chloride ion concentration was adjusted by replacing sodium chloride with sodium gluconate to give a constant sodium ion concentration. (C) The effect of pH was observed on PAH uptake mediated by NPT1. The closed and open symbols represent results obtained in HEK293 cells transfected with NPT1 and pcAGGS vector alone, respectively. The uptake was measured at pH 7.4 and 37°C for 0.5 min and each value is the mean and SEM of three determinations.

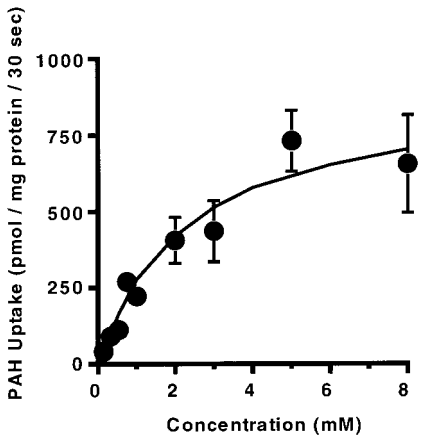


**FIG. 4.** Concentration dependence of [$^3$H]PAH uptake by human NPT1 expressed in HEK293 cells. [$^3$H]PAH uptake was measured in chloride ion-free medium. Uptake of PAH via NPT1 was estimated by subtraction of the uptake by mock-transfected cells from that by NPT1-transfected HEK293 cells. The uptake was measured at pH 7.4 and 37°C for 0.5 min and is shown as the mean and SEM of three determinations.

ing drugs. The NPT1-mediated transport of PAH was chloride ion-sensitive. In this study, we found no evidence of an anion exchange mechanism for PAH via NPT1 (Fig. 6). PAH uptake was not inhibited by 2-ketoglutaric acid (Table 1). Rabbit NaPi1, a homologue of human NPT1, mediates electrogenic transport of benzylpenicillin (12). The $K_m$ value of PAH transport via NPT1 (2.66 mM) (Fig. 4) was consistent with reported values of the classical PAH transport system (0.8 mM and 5.8 mM) on the renal apical membrane (20, 21). Therefore, although we have no evidence for membrane potential dependence of PAH transport via NPT1 in the present study, PAH transport via NPT1 could be the voltage-sensitive pathway.

NPT1-mediated transport of PAH was chloride ion-sensitive. Accordingly, a chloride ion gradient, as well as the membrane potential, could affect the direction of net PAH transport. Because a concentration gradient of chloride ions exists between intracellular and extracellular fluids (22), PAH transport may be secretory.

As has been demonstrated in the present study, human NPT1 recognized PAH, probenecid, $\beta$-lactam antibiotics and salicylic acid (Table 1). The substrate specificity of NPT1 largely overlapped with that of OAT1, the basolateral organic anion transporter (4, 5). Since NPT1 is localized at the apical membrane of renal proximal tubular cells (10), it is expected that OAT1 transports organic anions into proximal epithelial tubular cells and NPT1 transports them from the cells to the urine.

Busch *et al.* reported that rabbit NaPi-1 did not stimulate PAH uptake (12). However, we showed that human NPT1 transports PAH and several other anions such as uric acid, $\beta$-lactam antibiotics (benzylpenicillin and faropenem), a glucuronide conjugate (estradiol-17$\beta$-glucuronide) and indomethacin. Moreover, we observed that mouse Npt1 also exhibited PAH transport activity (unpublished observation). These functional differences may be ascribed to species difference. From the renal toxicological point of view, the organic anion transport system at the apical membrane is important since nephrotoxic drugs may be accumulated in the proximal tubular cells. Human NPT1 may be useful as a screening system to predict drug accumulation in renal epithelial cells, and to evaluate nephrotoxicity of newly developed drugs.

Previously, we had identified human NPT1 as a sodium-dependent transporter for inorganic phosphate (9). We also found that the Type II Na/Pi transporter was the major contributor to phosphate reabsorption in the kidney (11). These observations indicated that the Type I Na/Pi transporter may have another role in renal proximal tubules. Busch *et al.* and we reported that some organic anions were transported via rabbit NaPi-1 and mouse Npt1 (12–15). In the present study, we examined the effect of phosphate ion on PAH uptake via human NPT1. Previously we reported that $K_m$ value of phosphate uptake via human NPT1 was 0.29 mM (9). If NPT1 shares the binding site of inorganic phosphate and organic anions, it would be expected that PAH uptake is inhibited by the 5 mM inorganic phosphate. As shown in Table 1, phosphate ion did not affect on [$^3$H]PAH uptake. Moreover, we confirmed that $^{32}$P-phosphate uptake at the concentration of 100 $\mu$M via NPT1 was 644 ± 66 pmol/mg protein/30 s.

**TABLE 1**

Inhibitory Effects of Various Compounds on [$^3$H]PAH Uptake by Human NPT1-Transfected Cells

| Inhibitor | Concentration (mM) | Relative uptake (% of control) |
|---|---|---|
| Control | — | 100.0 |
| PAH | 5 | 26.4 ± 17.18* |
| Uric acid | 1 | 82.2 ± 19.02 |
| Benzylpenicillin | 5 | 3.1 ± 16.56* |
| Faropenem | 5 | 34.4 ± 11.04* |
| Indomethacin | 1 | 5.1 ± 2.96* |
| Probenecid | 1 | 51.5 ± 9.82* |
| Salicylic acid | 5 | 26.4 ± 19.02* |
| DIDS | 0.1 | 35.0 ± 5.52* |
| | 1 | 25.2 ± 1.84* |
| Lactic acid | 5 | 90.8 ± 16.56 |
| Phosphate | 5 | 89.8 ± 12.94 |
| Succinic acid | 5 | 117.8 ± 17.18 |
| 2-Ketoglutaric acid | 5 | 107.4 ± 12.27 |

*Note.* Uptake was measured for 30 s in chloride ion-free medium as described in the legend to Fig. 2 in the absence or presence of 5 mM inhibitor. These data were obtained by subtraction of the uptake by control DNA-transfected cells from that by NPT1-transfected cells.

* Significantly different from the control uptake by Student's *t* test ($P < 0.05$).

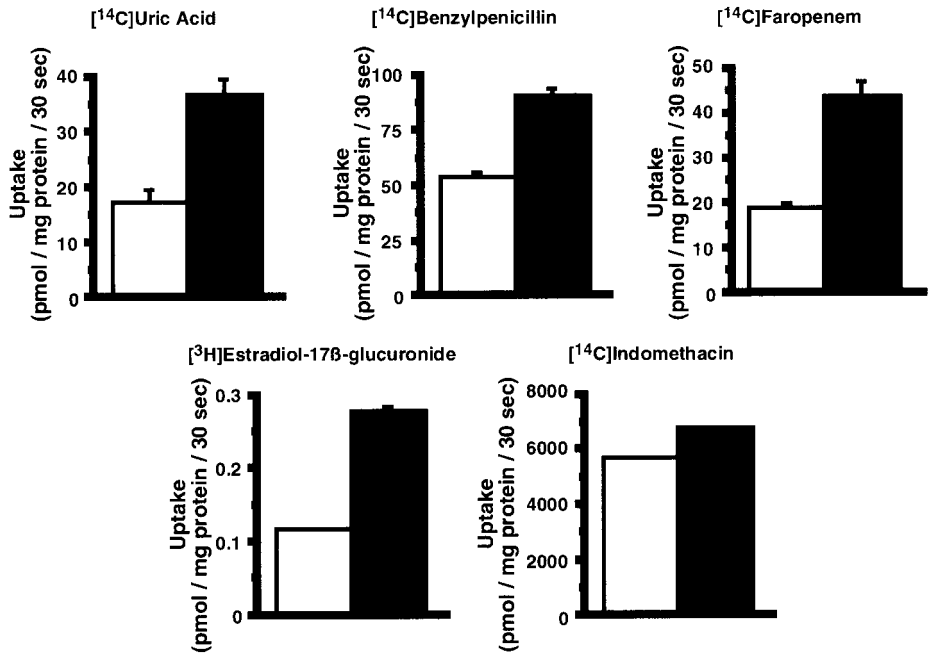


**FIG. 5.** Stimulation of transport of various organic anions by human NPT1 expressed in HEK293 cells. Uptakes of various compounds were tested at pH 7.4 and 37°C for 0.5 min in chloride ion-free medium. The closed and open bars represent the uptake by HEK293 cells transfected with NPT1 and with pCAGGS plasmid alone, respectively. The concentrations of radiolabeled substrates were 10 $\mu$M [$^{14}$C]uric acid, 9 $\mu$M [$^{14}$C]benzylpenicillin, 10 $\mu$M [$^{14}$C]faropenem, 9 nM [$^{3}$H]estradiol-17$\beta$-glucuronide and 22 $\mu$M [$^{14}$C]indomethacin. Each result is the mean and SEM of three determinations.

Therefore, we concluded that human NPT1 transports inorganic phosphate and organic anions in the distinct manners, and NPT1 seemed to have at least two independent roles, reabsorption of phosphate and secretion of organic anions, in renal proximal tubules.

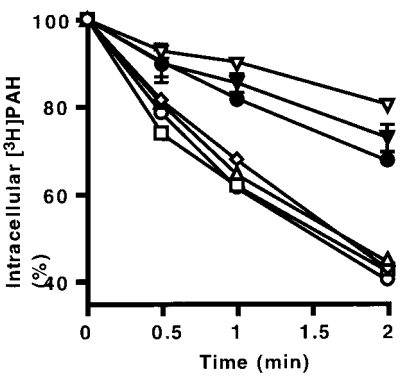


**FIG. 6.** Effect of extracellular ions on [$^{3}$H]PAH efflux from HEK293 cells expressing human NPT1. Efflux of preloaded [$^{3}$H]PAH was measured in mock and human NPT1-transfected cells. The cells were preincubated with [$^{3}$H]PAH for 10 min at the concentration of 100 $\mu$M and subsequently used for efflux study by suspending the cells with medium containing the test compounds. Open circles, squares, triangles, diamonds and upside-down triangles represent the remaining amount of [$^{3}$H]PAH in NPT1-expressing cells in the presence of gluconate (control), PAH, chloride, inorganic phosphate and indomethacin, respectively, in the extracellular medium. Closed circles and upside down triangles represent the amount in mock cells in the presence of gluconate and indomethacin, respectively. Efflux studies were performed at pH 7.4 and 37°C and the anion concentrations were 1 mM PAH, 133 mM chloride, 1 mM phosphate, and 1 mM indomethacin. Each result is the mean and SEM of three determinations.

Recently, Wu *et al.* and we have found that the organic cation transporter OCTN2 is also a bifunctional transporter, i.e., it transports carnitine and organic cations in sodium-dependent and sodium-independent manners, respectively (19, 23, 24). Such multifunctionality of the transporter might be advantageous in terms of transport efficiency.

In conclusion, we have identified the PAH transporter on the renal apical membrane. NPT1 exists on the apical membrane of the renal proximal tubules and transports various organic anions, including PAH, uric acid, glucuronide conjugate and $\beta$-lactam antibiotics, in a chloride-ion sensitive manner. Although the physiological relevance of NPT1 in organic anion transport is still unclear, this finding should contribute to our understanding of the renal secretion mechanisms of organic anions at the molecular level.

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