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Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions

(phosphate/transport/ion channels)

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ABSTRACT **Two distinct molecular types (I and II) of renal proximal tubular brush border Na^+/P_i cotransporters have been identified by expression cloning on the basis of their capacity to induce Na^+-dependent P_i influx in tracer experiments. Whereas the type II transporters (e.g., NaPi-2 and NaPi-3) resemble well known characteristics of brush border Na^+/P_i cotransport, little is known about the properties of the type I transporter (NaPi-1). In contrast to type II, type I transporters produced electrogenic transport only at high extracellular P_i concentrations (≥3 mM). On the other hand, expression of NaPi-1 induced a Cl^- conductance in *Xenopus laevis* oocytes, which was inhibited by Cl^- channel blockers [5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) > niflumic acid ≫ 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid]. Further, the Cl^- conductance was inhibited by the organic anions phenol red, benzylpenicillin (penicillin G), and probenecid. These organic anions induced outwardly directed currents in the absence of Cl^-. In tracer studies, we observed uptake of benzylpenicillin with a K_m of 0.22 mM; benzylpenicillin uptake was inhibited by NPPB and niflumic acid. These findings suggest that the type I Na^+/P_i cotransporter functions also as a novel type of anion channel permeable not only for Cl^- but also for organic anions. Such an apical anion channel could serve an important role in the transport of Cl^- and the excretion of anionic xenobiotics.**

Renal proximal tubule cells play a major role in the reabsorption and excretion of physiological substrates as well as xenobiotics. Two proteins with distinct molecular structures (type I and II) have been identified in the apical membrane of renal tubule cells and shown to induce transport of P_i upon expression in *Xenopus* oocytes (1–4). The type II Na^+-coupled P_i transporter expressed in *Xenopus* oocytes meets all known characteristics of renal brush border membrane Na^+/P_i cotransport, such as sigmoidal Na^+ dependence, pH dependence, high P_i affinity (K_m of 0.1 mM), and regulation by protein kinase C (2, 5–7). This transporter is altered at the functional and molecular levels under a variety of physiological and pathophysiological conditions (8–12). In contrast to type II, the type I transporter induces only a relatively weak P_i-transport activity with a low P_i affinity in *Xenopus* oocytes (1). In further contrast to the type II transporter, the type I protein lacks another main characteristic of P_i transport, namely an increase of its expression during a low P_i diet (3, 8, 13). Therefore, we were interested in the transport characteristics of the type I P_i transporter, its electrogenic properties of transport, and the question of whether it may serve another function in the apical membrane of renal proximal tubule cells.

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MATERIALS AND METHODS

cRNA encoding NaPi-1 (1), NaPi-2 (2), NaPi-3 (2), and rBAT (14) was synthesized *in vitro* as previously described. Dissection of *Xenopus laevis* ovaries and collection and handling of the oocytes have been described (15). The experiments were performed on oocytes injected with 10 ng of cRNA per oocyte. Two-electrode voltage-clamp recordings were performed 3–8 days after injection at room temperature using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) and a MacLab D/A converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The external control solution (ND 96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes (titrated with NaOH to pH 7.4). For some experiments, all extracellular cations were substituted with tetraethylammonium (TEA; 100 mM) or Ca^{2+} was omitted (ND 96/0 Ca^{2+}). In some experiments, all external Cl^- was substituted by gluconate, iodide, or bromide. Because of its Ca^{2+}-chelating capacity, the free Ca^{2+} concentration in the gluconate concentration was <0.1 mM. Replacement of Cl^- with gluconate caused a junction potential of ≈10 mV that must be considered for the interpretation of the shift in reversal potentials and depolarizations observed after the substitution of Cl^- with gluconate (see *Results and Discussion*). The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s. Uptake studies with benzyl[^{14}C]penicillin and [^{3}H]*p*-aminohippurate (PAH) were performed as described (16). Chemicals used were 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB; a gift from R. Greger, University of Freiburg, Germany), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), niflumic acid, phenol red, benzylpenicillin and probenecid (Sigma), and benzyl[^{14}C]penicillin (Amersham). PAH and [^{3}H]PAH were a generous gift from K. J. Ullrich (Max Planck Institute, Frankfurt, Germany). All data are given as mean ± SEM, with *n* indicating the number of experiments. The recorded currents in NaPi-1-expressing oocytes varied significantly, depending on the time period after cRNA injection and on the batch of oocytes (from different animals). Therefore, throughout the paper, we show experimental data obtained on one specific day. All sets of experiments have been repeated at least once; in all repetitions, qualitatively similar data have been obtained.

RESULTS AND DISCUSSION

As described in detail below, large inwardly directed holding currents were required to clamp *Xenopus* oocytes expressing

Abbreviations: TEA, tetraethylammonium; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; PAH, *p*-aminohippurate.

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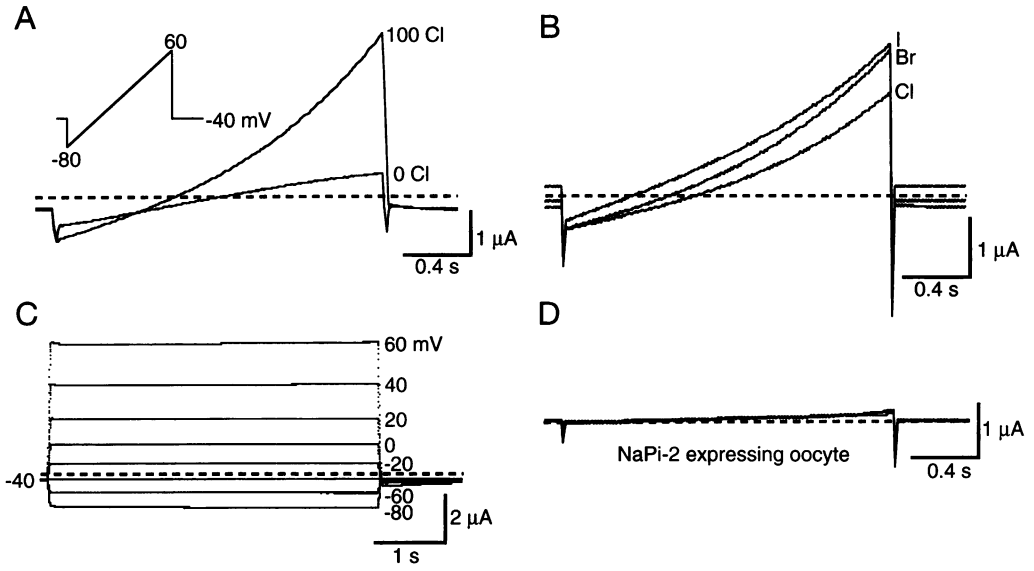

FIG. 1. Currents in *Xenopus* oocytes induced by the expression of the type I Na^+/P_i cotransporter NaPi-1 (*A–C*) and the type II transporter NaPi-2 (*D*). In *A*, *B*, and *D*, currents were recorded during voltage ramps (duration was 2 s) from −80 to 60 mV (holding potential was −40 mV). The dashed line indicates 0 current. (*A*) Replacement of extracellular Cl^- with gluconate (0 Cl) reduces the outwardly rectifying current and shifts the reversal potential (potential at 0 current) to the right. (*B*) Replacement of extracellular Cl^- with iodide (I) or bromide (Br) increases the conductance and shifts the reversal potential to the left. (*C*) The currents induced by NaPi-1 did not inactivate during long voltage steps (5 s) to the voltages indicated at the current traces. (*D*) In NaPi-2-expressing oocytes, no significant background current was observed. This panel shows superimposed traces under the same conditions as described for *B*.

NaPi-1 at a holding potential of −50 mV, but superfusion with P_i at concentrations <3 mM did not induce significant currents deviating from the holding current. Superfusion with 1 mM P_i induced inward currents of −3.6 ± 1.8 nA (n = 20) and 1.8 ± 0.3 nA (n = 6) in NaPi-1-expressing and control oocytes, respectively (P = 0.595). This result is in contrast to results obtained with the type II Na^+/P_i cotransporters NaPi-2 and NaPi-3, which did not induce a background conductance under control conditions but induced Na^+/P_i-carried currents at low extracellular P_i concentrations (5, 6). In NaPi-1-expressing oocytes, only P_i concentrations ≥3 mM produced an inward current that was larger than the current induced in H_2O-injected oocytes (−14.2 ± 3.5 nA vs. −2.3 ± 0.5 nA; n = 6 and 5, respectively). This current was partially Na^+ dependent and reduced to −5.5 ± 3.7 nA (n = 6) in the absence of extracellular Na^+. The current difference of ≈10 nA corresponds to a net inward movement of 6 pmol of positive charges per min, which corresponds approximately to the Na^+-dependent P_i transport in previous studies on NaPi-1-expressing oocytes (1).

However, as mentioned above, the expression of NaPi-1 induced in all oocytes (n = 220) a background conductance ranging from 2.5 μS to 200 μS (calculated from currents elicited with voltage ramps from −80 to 60 mV), depending on the batch of oocytes and the day of NaPi-1 expression. In one batch of oocytes, the NaPi-1-induced conductance was 19.4 ± 3.7 μS (n = 7) and 167.9 ± 9.3 μS (n = 7) on days 2 and 6 of NaPi-1 expression, respectively. The reversal potential for this conductance was −30.3 ± 0.8 mV, which corresponds to the equilibrium potential for Cl^- (E_{Cl}) in *Xenopus* oocytes (Fig. 1*A*; n = 30). The NaPi-1-induced conductance was outwardly rectifying (i.e., at more positive potentials, the currents per voltage difference were larger than at negative voltages).

This conductance was independent of extracellular Ca^{2+}. When extracellular Ca^{2+} was omitted (ND 96/0 Ca^{2+}), the conductance was 21.0 ± 1.4 μS (n = 5) compared with 20.1 ± 1.3 μS (n = 5) in the presence of 1.8 mM extracellular Ca^{2+} (ND 96). Furthermore, a complete replacement of extracellular cations with TEA (100 mM) did not significantly alter this conductance. Under control and TEA, the NaPi-1-induced

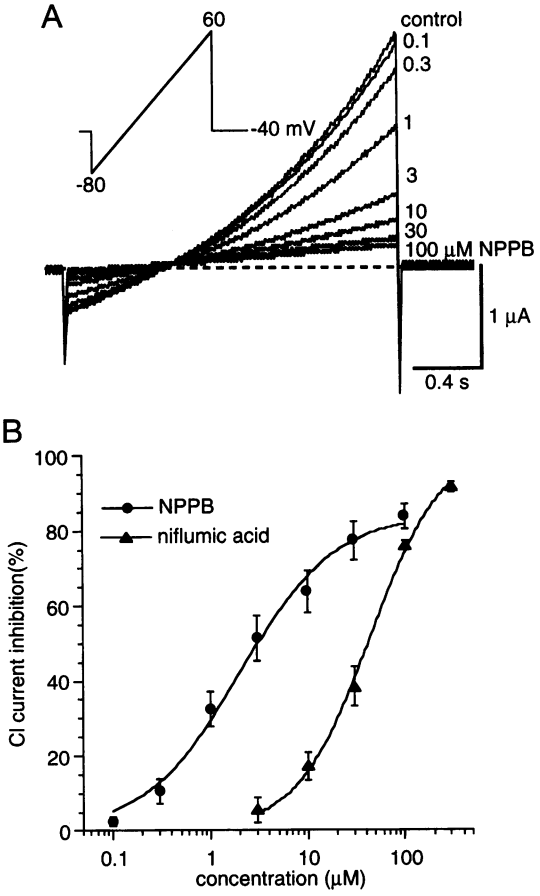

FIG. 2. Inhibition of NaPi-1-induced Cl^- currents by Cl^- channel blockers. (*A*) NPPB inhibits, depending on the concentration, both inward and outward currents. (*B*) Concentration-inhibition relationship for NPPB and niflumic acid for the current at −80 mV. NPPB displays ≈10-fold higher affinity compared with niflumic acid.

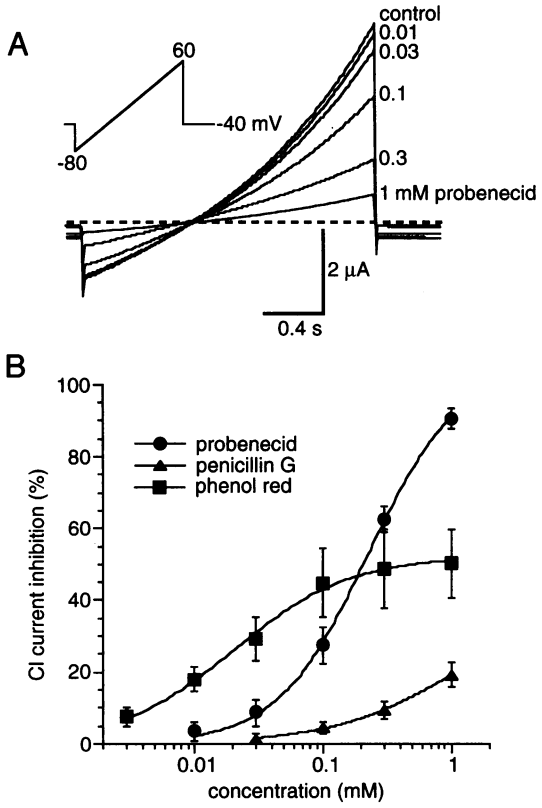

FIG. 3. Inhibition of NaPi-1-induced Cl^- currents by organic anions. (*A*) Probenecid-mediated Cl^- current inhibition is almost complete at 1 mM. (*B*) Concentration-inhibition relationship for probenecid, benzylpenicillin (penicillin G), and phenol red. Penicillin G is the weakest inhibitor, whereas phenol red displays the highest apparent affinity but a maximal inhibition of only ≈50%.

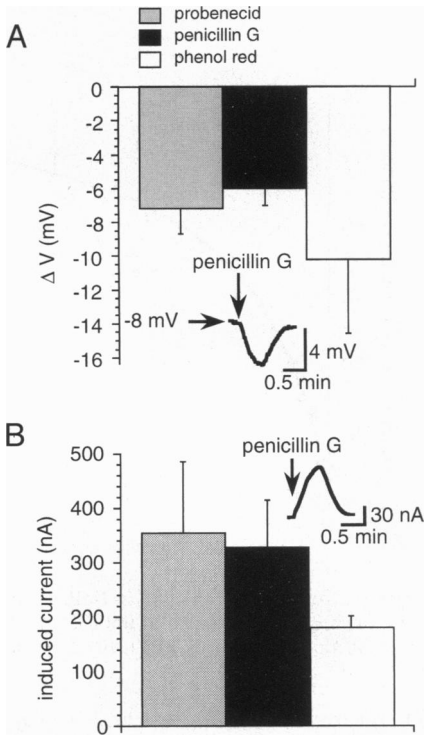

FIG. 4. Effects of the organic anions probenecid, penicillin G, and phenol red in the absence of Cl^-. In current-clamp (*A*) and voltage-clamp (*B*; holding potential was −80 mV) conditions, all three anions induce a hyperpolarization and outward currents, respectively. The traces display the effects of a 30-s superfusion period with penicillin (1 mM).

conductance was 9.2 ± 1.7 μS (n = 6) and 8.4 ± 1.4 μS (n = 6), respectively.

Substitution of extracellular Cl^- with gluconate dramatically decreased both inward and outward currents and shifted the reversal potential to −11.9 ± 5.6 mV (Fig. 1*A*; n = 9), strongly suggesting that the observed currents are carried by Cl^-. Considering the liquid junction potential caused by the replacement of Cl^- with gluconate (≈10 mV) the actual shift of the reversal potential is even higher. The conductance was also permeable for bromide and iodide, which shifted the reversal potential from −30.8 ± 1.6 mV at control to −39.7 ± 1.7 mV and −52.9 ± 1.5 mV (n = 6), respectively (Fig. 1*B*). At 60 mV, both Br^- and Cl^- with 3.12 ± 0.31 μA and 2.98 ± 0.24 μA, respectively, induced a significantly larger outward current than Cl^- (2.30 ± 0.22 μA; n = 5). The conductance was permanently open and did not inactivate during 5-s depolarizations or hyperpolarizations (Fig. 1*C*; n = 6).

Oocytes expressing the type II Na^+/P_i transporters NaPi-2 or NaPi-3 (2) induced inward currents upon superfusion with P_i (1 mM) as described (5, 6) but did not induce a conductance in the absence of P_i (Fig. 1*D*). At day 6 after RNA injection, the conductance in NaPi-2- and NaPi-3-expressing oocytes was 1.4 ± 0.2 μS (n = 25) and 1.4 ± 0.1 μS (n = 15), respectively, in the absence of extracellular P_i. Furthermore, expression of the amino acid exchanger rBAT, another renal brush border transporter, induced outward and inward currents upon superfusion with L-leucine (1 mM) or L-arginine (0.1 mM) as described (17) but did not induce a conductance in the absence of neutral or dibasic amino acids (the conductance was 1.8 ± 0.2 μS at day 6 of rBAT expression; n = 6). Moreover, a permanently open Cl^- conductance could also not be detected in H_2O-injected or noninjected oocytes. The conductances here were 1.6 ± 0.1 μS (n = 16) and 1.2 ± 0.1 μS (n = 35) for H_2O-injected and noninjected oocytes, respectively, suggesting that NaPi-1 specifically induces the anion conductance described above.

The NaPi-1-induced Cl^- conductance was sensitive to Cl^- channel blockers (Fig. 2 *A* and *B*). NPPB and niflumic acid inhibited NaPi-1-induced currents effectively with EC_{50} values of 1.99 ± 0.22 μM (n = 5) and 43.0 ± 12.6 μM (n = 5), respectively, whereas DIDS was a much weaker blocker (45.5 ± 4.1% inhibition at 1 mM; n = 8).

In the apical membrane of renal proximal tubule cells, the site of NaPi-1 expression (18, 19), numerous anion transport systems and/or conductances have been described, one of which is permeable to organic anions and inhibited by probenecid (20). Therefore, we analyzed the effects of the organic anions probenecid, phenol red, and benzylpenicillin on NaPi-1-induced Cl^- currents. These three organic anions inhibited the Cl^- conductance with distinct potencies (Fig. 3 *A* and *B*). The maximal inhibition of the Cl^- conductance by probenecid was extrapolated to 100% with an EC_{50} of 0.22 ± 0.03 mM (n = 6). The maximal extrapolated inhibition with phenol red and benzylpenicillin was 52.3 ± 1.7% (n = 6) and 43.1 ± 19.4% (n = 5) at EC_{50} values of 0.02 ± 0.01 mM and 1.28 ± 0.14 mM, respectively. In contrast to these compounds, the organic anion PAH had at 1 mM no effect on the NaPi-1-induced conductance. The conductance was 40.2 ± 5.5 μS (n = 5) and 41.9 ± 5.4 μS (n = 5) under control and 1 mM PAH, respectively.

The effects of organic anions on NaPi-1-expressing oocytes were then studied under distinct conditions. In current-clamp experiments, the control membrane potential was −33.6 ± 3.5 mV (n = 14). Under these conditions, probenecid and phenol red (each at 1 mM) and benzylpenicillin (10 mM) had no effects on the membrane potential, which was −34.2 ± 1.2 mV (n = 6), −34.8 ± 1.8 mV (n = 4), and −35.2 ± 2.1 mV (n = 4), respectively, upon superfusion with these compounds.

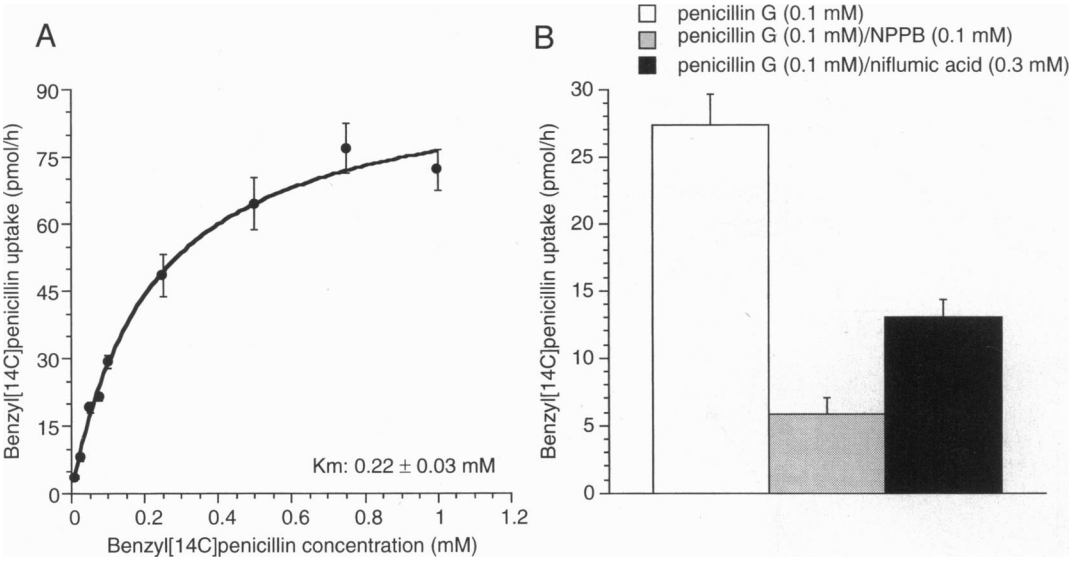

FIG. 5. Uptake of benzyl[^{14}C]penicillin in NaPi-1-expressing oocytes. Incubation time was 15 min; the uptake was calculated in pmol/h after the subtraction of benzyl[^{14}C]penicillin uptake in H_2O-injected oocytes. For each point, n was 7. (*A*) Concentration-dependence of benzyl[^{14}C]penicillin uptake fitted by Michaelis–Menten kinetics. (*B*) Benzyl[^{14}C]penicillin uptake in the absence and presence of NPPB and niflumic acid.

However, when extracellular Cl^- was replaced by gluconate, these compounds caused a hyperpolarization. Probenecid, phenol red, and benzylpenicillin (all at 1 mM) changed the resting membrane potential in the absence of extracellular Cl^- (the measured control potential under gluconate was -13.8 ± 4.8 mV; $n = 14$) by -7.2 ± 1.5 mV ($n = 6$), -10.2 ± 4.4 mV ($n = 4$), and -6.1 ± 3.3 mV ($n = 4$), respectively (Fig. 4*A*). In voltage-clamp experiments (at -80 mV), probenecid ($n = 6$), phenol red ($n = 4$), and benzylpenicillin induced in the absence of extracellular Cl^- outward currents (see Fig. 4*B*).

The appearance of a hyperpolarization or outward currents could be either the result of a direct inward movement of these organic anions or the inhibition of a residual outward movement of an anion (i.e., inhibition of an inward current). Extended superfusion periods (>10 min) with Cl^- free solution at a holding potential of -80 mV did not alter the holding current and probenecid (1 mM) -induced outward current ($n = 6$). The inhibition of a residual outward movement of Cl^- or another endogenous anion is, therefore, unlikely to be the main reason for the probenecid-induced hyperpolarization. Therefpre, we tested the only available radioactive of these three anions, benzyl[^{14}C]penicillin, for uptake. NaPi-1-expressing oocytes indeed displayed benzyl[^{14}C]penicillin uptake that saturated with an apparent K_m of 0.22 ± 0.03 mM (Fig. 5*A*; $n = 7$). Benzyl[^{14}C]penicillin (0.1 mM) uptake was reduced to 21.4% and 48.8% of control in the presence of NPPB (0.1 mM) and niflumic acid (0.3 mM), respectively (Fig. 5*B*; $n = 7$). In contrast, NaPi-1 expression did not increase uptake of [^{3}H]PAH (0.1 mM) into oocytes. Extrapolated uptake of [^{3}H]PAH was 22.3 ± 1.3 pmol/h ($n = 7$) and 20.5 ± 1.7 pmol/h ($n = 7$) in NaPi-1-expressing and H_2O-injected oocytes, respectively ($P = 0.417$).

The data presented here demonstrate that NaPi-1 induces an anion conductance upon expression in *Xenopus* oocytes. So far, induction of anion conductances in *Xenopus* oocytes could be shown for representatives of the ClC family (21), CFTR (22), and I_{Cln} (23), a protein that induces a swelling-induced Cl^- conductance upon expression in *Xenopus* oocytes. NaPi-1 shows no molecular similarities to any of these proteins. Moreover, all these conductances can be clearly distinguished from the NaPi-1-induced anion conductance reported in this study by electrophysiological means (voltage dependence of activation, inactivation, and deactivation) by their anion selectivity (rank order of permeability for Cl^-, Br^-, and I^-) and pharmacology (sensitivity for the Cl^- channel blocker NPPB, niflumic acid, and DIDS). To our knowledge, there has been no report on an anion conductance in *Xenopus* oocytes (endogenous or expressed after cRNA injection) that meets the characteristics of the conductance induced by NaPi-1 (i.e., permanently activated, outwardly rectifying anion conductance, high I^- permeability, and high NPPB but low DIDS sensitivity). Moreover, the expression of a number of unrelated membrane proteins (type II P_i transporters NaPi-2 and NaPi-3 and the amino acid exchanger rBAT) did not induce a similar conductance nor was such a conductance ever observed in noninjected or H_2O-injected oocytes, suggesting that this anion conductance is specifically induced by NaPi-1. NaPi-1, which was originally cloned from rabbit kidney and is also highly expressed in liver, displays no molecular similarities to any of the proteins known to induce anion conductances in *Xenopus* oocytes. However, the human analog gene, which is almost identical to NaPi-1, was recently cloned (24). Moreover, a brain-specific protein related to NaPi-1 (BNPI) was recently identified (25). If BNPI induces also an anion conductance, its influence on neuron excitability would be of high physiological importance. In kidney, NaPi-1 may play an important role both in the Cl^- balance of proximal tubule cells and for the excretion of anionic xenobiotics. A blockade of NaPi-1 (e.g., with probenecid) could increase the half-lives of other anionic xenobiotics (e.g., benzylpenicillin).

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