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Electrophysiological analysis of Na^+/P_i cotransport mediated by a transporter cloned from rat kidney and expressed in *Xenopus* oocytes

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ABSTRACT **Phosphate (P_i) reabsorption in renal proximal tubules involves Na^+/P_i cotransport across the brush border membrane; its transport rate is influenced by the Na^+-coupled transport of other solutes as well as by pH. In the present study, we have expressed a cloned rat renal brush border membrane Na^+/P_i cotransporter (NaPi-2) in *Xenopus laevis* oocytes and have analyzed its electrophysiologic properties in voltage- and current-clamp studies. Addition of P_i to Na^+-containing superfusates resulted in a depolarization of the membrane potential and, in voltage-clamped oocytes, in an inward current (I_P). An analysis of the Na^+ and/or P_i concentration dependence of I_P suggested a Na^+/P_i stoichiometry of 3:1. I_P was increased by increasing the pH of the superfusate; this phenomenon seems to be mainly related to a lowering of the affinity for Na^+ interaction by increasing H^+ concentration. The present data suggest that known properties of P_i handling at the tubular/membrane level are "directly" related to specific characteristics of the transport molecule (NaPi-2) involved.**

Renal proximal tubular P_i reabsorption involves brush border membrane Na^+/P_i cotransport. Rates of proximal tubular P_i reabsorption and of brush border membrane Na^+/P_i cotransport are under physiological control, involving a variety of hormonal and nonhormonal control mechanisms (for review, see refs. 1–3). Of particular interest in the context of the present study are two phenomena in proximal tubular P_i handling: (*i*) in most species proximal tubular P_i reabsorption and brush border membrane Na^+/P_i cotransport are increased by increasing intratubular/extravesicular pH (for review, see refs. 1–3; for examples, see refs. 4–7) and (*ii*) parallel operation of other Na^+-coupled transport pathways reduces the rates of tubular P_i reabsorption and of brush border membrane Na^+/P_i cotransport (for example, see refs. 8 and 9).

Renal Na^+/P_i cotransport has been extensively characterized in studies on cortical brush border membrane vesicles (for review, see refs. 1–3; for examples, see refs. 5–7 and 9–17); these studies provided evidence for a Na^+/P_i stoichiometry exceeding unity and that the pH dependence of tubular P_i reabsorption might be related to multiple factors, among them strong pH effects on Na^+ interaction (5) and some preferential transport of divalent P_i (6, 7, 10, 11). Kinetic studies on brush border membrane Na^+/P_i cotransport provided evidence for a heterogeneity/multiplicity of brush border membrane Na^+/P_i cotransport systems (16, 17).

Rabbit (NaPi-1; ref. 18), rat (NaPi-2; ref. 19), and human (NaPi-3; ref. 19) renal Na^+/P_i cotransporters have been identified by using the *Xenopus laevis* expression cloning system; the proximal tubular and brush border location of NaPi-1- and NaPi-2-related transport systems could be documented at the mRNA as well as at the protein level (20–22). NaPi-1 is not homologous to NaPi-2 or NaPi-3, but the latter two are highly homologous to each other (18, 19). Although, NaPi-1 and NaPi-2/3 genes have not been identified within the same species, they might be related to the above-mentioned heterogeneity/multiplicity of tubular Na^+/P_i cotransporters. Brush border membrane Na^+/P_i cotransport in the kidney seems to be different from brush border membrane Na^+/P_i cotransport in the small intestine; most strikingly, the rate of small-intestine Na^+/P_i cotransport is increased by lowering of extravesicular pH (23). Also, use of molecular tools such as cDNA probes and specific antibodies gave no evidence for a structural similarity between renal and intestinal Na^+/P_i cotransporters (J.B. and H.M., unpublished work).

The above-mentioned tools have been used to study physiological regulation of renal Na^+/P_i cotransport. Dietary P_i deprivation led to an increase in content of specific mRNA and protein of NaPi-2 (rat) (ref. 24; J.B. and H.M., unpublished work) but not of NaPi-1 (25). Further, the content of NaPi-2-related mRNA and protein is reduced in accordance with reduced transport activity in a hypophosphatemic mouse model (*Hyp* mouse; ref. 26). These observations suggest that NaPi-2-related transport activity is physiologically regulated and determines mainly the rate of proximal tubular P_i reabsorption.

To characterize further NaPi-2-related transport activity and to further support its important role in brush border membrane Na^+/P_i cotransport, we have performed an electrophysiological analysis of Na^+/P_i cotransport after expression of NaPi-2 in *X. laevis* oocytes. We have been able to document an electrogenic behavior of this transport activity and a strong pH dependence. We conclude that Na^+/P_i cotransport involves at least a stoichiometry of 3:1 for Na^+ versus P_i. Further, the pH dependence seems to relate mainly to an effect of pH on Na^+ interaction. The observed electrophysiological characteristics are in complete agreement with the overall behavior of P_i transport at the tubular as well as at the brush border membrane level.

METHODS

cRNA encoding NaPi-2 was synthesized *in vitro* as described (18, 27). Dissection of *X. laevis* ovaries and collection and handling of the oocytes have been described in detail (28). If not otherwise indicated, the experiments were performed on oocytes injected with 10 ng of cRNA per oocyte. Two-electrode current- and voltage-clamp recordings were performed 3–8 days after injection at room temperature (29). In

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Abbreviation: I_P, P_i-induced current.

voltage- and current-clamp experiments the oocytes were clamped at −50 mV and zero current, respectively, if not otherwise stated. The data were filtered at 10 Hz and recorded on a chart recorder. The external solution (superfusate) contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes. P_i was added to this solution at the indicated concentrations. The final solutions were titrated to the indicated pH with HCl or NaOH. To study the Na^+ dependence of P_i-induced current, NaCl was partially replaced by choline chloride. In those experiments KOH was used instead of NaOH for titration. The flow rate of the superfusion was 10 ml/min and a complete exchange of the bath solution was reached within about 15 sec. The currents or depolarizations stated are the maximal values measured during a 30-sec substrate superfusion; as P_i transport was found to be associated with a net inward movement of positive charge, the resulting current was given a negative (−) sign. All data are given as means ± SEM, where *n* indicates the number of experiments. The size of the P_i-induced current (I_P) varied 2- to 3-fold, depending on the time period after cRNA injection and on the different batches of oocytes (from different animals). Therefore, throughout the paper we show data obtained on the same day for a specific set of experiments on multiple oocytes derived from one frog. All experiments have been repeated with two or three batches of oocytes; in all repetitions, qualitatively similar data have been obtained.

RESULTS AND DISCUSSION

Electrophysiological Characterization of Na^+/P_i Cotransport. In current-clamp studies (zero current was applied) on oocytes injected with 10 ng of cRNA, application of 1 mM P_i resulted in a depolarization of the oocyte membrane potential from −43.7 ± 2.7 mV to −34.0 ± 1.6 mV at pH 7.3 (n = 4), indicating the movement of positive charges into the cell.

In voltage-clamp experiments (holding potential of −50 mV), addition of 1 mM P_i to extracellular fluid induced a net inward current (I_P) across the cell membrane of oocytes previously injected with cRNA encoding for NaPi-2. Thus, the transport of P_i was paralleled by movement of net positive charges. I_P was dependent on the amount of cRNA injected. In water-injected oocytes, 1 mM P_i induced a current of −0.3 ± 0.1 nA (n = 6; Fig. 1). Four days after injection, 1 mM P_i induced a current of −5.7 ± 0.7 nA (n = 5) in oocytes injected with 1 ng of cRNA, and a current of −52.7 ± 4.1 nA (n = 6) in oocytes injected with 10 ng of cRNA at a holding potential of −50 mV. For further analysis only I_P was analyzed and only oocytes injected with 10 ng of cRNA were used.

Electrogenic transport is expected to be dependent on the electrical driving force and thus on the electrical potential difference across the oocyte plasma membrane. As illustrated in Fig. 2, I_P was indeed a function of this electrical potential. Changing the potential difference from −80 to 10 mV decreased I_P from −34.0 ± 3.6 nA to −13.1 ± 1.0 nA (n = 4). A similar potential dependence has been shown previously for other Na^+-coupled transporters (30–33).

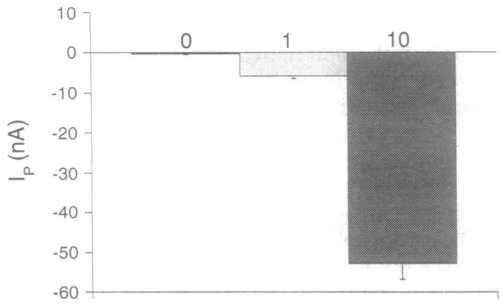

FIG. 1. Amplitudes of I_P in *Xenopus* oocytes injected with either water (0) or 1 or 10 ng of cRNA encoding NaPi-2. Holding potential was −50 mV and pH was 7.5. I_P was induced by superfusion of 1 mM P_i for 30 sec. Data are given as means and SEM for n = 6, 5 and 6, respectively.

Na^+ and P_i Interaction with NaPi-2. I_P was a function of extracellular Na^+ concentration (Fig. 3). Fitting the data to the Hill equation resulted in a half-maximal I_P at 58.2 ± 1.3 mM Na^+ with a Hill coefficient of 3.12 ± 0.02 at pH 7.3 (Fig. 3; n = 5). The Hill coefficient is compatible with the figure of 2.5 determined from tracer uptake studies in oocytes expressing NaPi-2 (19). I_P was also dependent on P_i concentration (Fig. 4). At pH 7.5 a fit with the Hill equation resulted in a maximal current of about −55.2 ± 3.3 nA and a half-maximal current at 0.31 ± 0.03 mM P_i (n = 5). The Hill coefficient for P_i was 1.14 ± 0.15. Both P_i and Na^+ affinities of I_P are similar to the affinity of P_i transport calculated from tracer uptake studies in oocytes expressing the same transporter (19). The Hill coefficients do suggest a 3:1 coupling ratio of Na^+ versus P_i.

pH Dependence of I_P. Na^+-dependent P_i reabsorption in the proximal tubule and Na^+/P_i cotransport across the brush border membrane are accelerated by increasing intratubular/extravesicular pH (see Introduction). Also, in the present study, I_P in oocytes expressing NaPi-2 was sensitive to ambient pH (Fig. 5). At 1 mM P_i and 100 mM Na^+, I_P decreased by about half following a decrease of pH from 7.8 to 6.3. It has been suggested earlier that the decreased Na^+/P_i cotransport at acidic pH in rat renal brush border membrane vesicles is mainly the result of reduced Na^+ affinity at high extravesicular H^+ concentration (5). As shown in Fig. 3, a decrease of pH to 6.3 in the superfusate was indeed paralleled by a shift of the apparent K_m to higher Na^+ concentrations. Clearly, Na^+ interaction at pH 6.3 can by far not reach saturation at 100 mM Na^+—i.e., at the maximal Na^+ concentration applicable without increasing superfusate osmolarity. For that reason, the extrapolated kinetical parameters at pH 6.3 can be taken only as crude estimates. Nevertheless, it is intriguing that application of the Hill equation yields an $I_{P(max)}$ and a Hill coefficient not significantly different from the respective values at pH 7.3 (see legend to Fig. 3). These

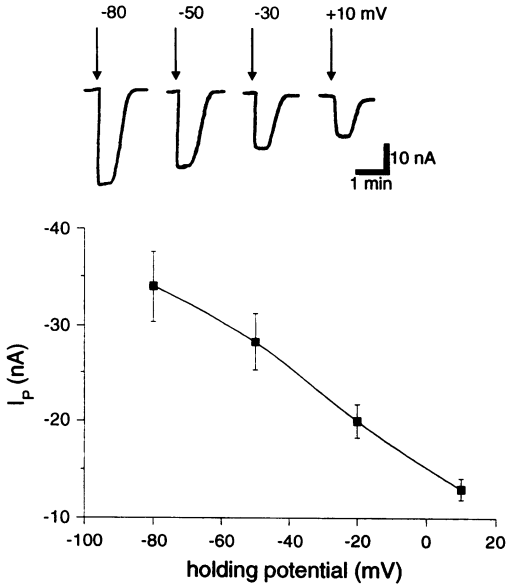

FIG. 2. I_P as a function of the potential difference across the cell membrane. (*Upper*) Original tracings of I_P at pH 7.3 for one characteristic oocyte. At the arrows 1 mM P_i was added for 30 sec at the holding potentials indicated. (*Lower*) Correlation between I_P and membrane potential (arithmetic means ± SEM, n = 4); the values for the maximal currents observed after superfusion with P_i were used.

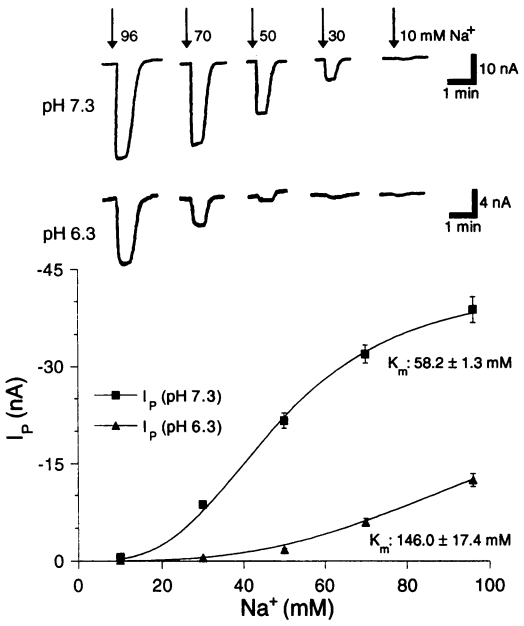

FIG. 3. Currents induced by 0.5 mM P_i as a function of ambient Na^+ concentration at a holding potential of −50 mV. To lower Na^+ concentration, Na^+ was isoosmotically replaced by choline. (*Upper*) I_P obtained at various Na^+ concentrations at pH 7.3 and 6.3 for one characteristic oocyte. Arrows indicate the start of a 30-sec P_i superfusion period in the presence of the Na^+ concentrations (mM) indicated above the traces. Note the different current scaling of the two panels. (*Lower*) Correlation between I_P and Na^+ concentration (arithmetic means ± SEM, $n = 5$ for each pH). The data were fitted by using the equation $I_P = I_{P(max)} \cdot [Na^+]^n/([Na^+]^n + K_m)$, where n and $[Na^+]$ give the Hill coefficient and the Na^+ concentration, respectively; $I_{P(max)}$ is the extrapolated maximal current; and K_m is the apparent concentration needed for half-maximal current. The calculated values were for $I_{P(max)}$, −43.0 ± 2.4 nA (pH 7.3) and −37.0 ± 5.0 nA (pH 6.3); for K_m, 58.2 ± 1.3 mM (pH 7.3) and 146 ± 17 mM (pH 6.3); and for n, 3.12 ± 0.02 (pH 7.3) and 3.04 ± 0.06 (pH 6.3). The extrapolated values at pH 6.3 can be taken only as crude estimates, since the carrier could not be saturated at 100 mM Na^+, the maximal concentration applicable at isotonic conditions.

extrapolated values suggest that the pH dependence of I_P might be abolished by increasing Na^+ concentration.

The above data are consistent with the conclusion from earlier studies on rat renal brush border membrane vesicles (5) that acidification impairs P_i transport mainly by decreasing the Na^+ affinity of the carrier. However, additional effects of pH on NaPi-2-mediated Na^+/P_i cotransport, such as on the protein itself or related to preferential transport of either mono- or divalent P_i, cannot be excluded. A consequence of acidification is a shift of P_i from the divalent to the monovalent form, and it has been suggested that divalent P_i is the preferred substrate for the carrier (6, 7, 10, 11). Given a pK of 6.8, the ratio of monovalent to divalent P_i is about 3:1 at pH 7.3, 1:1 at pH 6.8, and 1:3 at pH 6.3. If the preferential transport of divalent P_i is a major effect in the pH sensitivity of Na^+/P_i cotransport (6, 7, 10, 11), the observed alterations in I_P should follow a P_i-titration curve. This was not observed in the experiment presented in Fig. 5 (at 1 mM P_i); also at lower P_i concentrations the pH dependence of I_P did not follow the predictions made from P_i-titration (0.1 mM P_i at pH 7.5 and pH 6.5 induced I_P of −10.3 ± 0.8 nA and −11.8 ± 1.1 nA, respectively; $n = 5$). Earlier tracer uptake studies revealed a decline of P_i transport by 63% following a decrease of pH from 6.8 to 6.3 (19). I_P, however, declined only by 37% upon a decrease of pH from 6.8 to 6.3 at 1 mM P_i (Fig. 5). Thus, the charge carried per transported P_i apparently increases at acidic pH. Such considerations suggest that both monovalent P_i and divalent P_i are transported by the carrier.

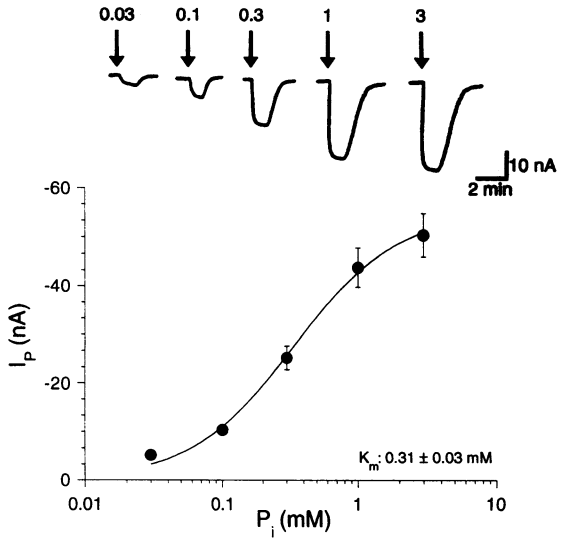

FIG. 4. P_i concentration dependence of I_P at pH 7.5 (holding potential, −50 mV). Correlation between current (arithmetic means ± SEM, $n = 5$) and P_i concentration is given by the graph. The data were fitted by using the equation $I_P = I_{P(max)} \cdot [P_i]^n/([P_i]^n + K_m)$, where n and $[P_i]$ give the Hill coefficient and the superfusate P_i concentration, respectively. Extrapolated $I_{P(max)}$ was −55.2 ± 3.3 nA.

Finally, the present data suggest strongly a 3:1 stoichiometry in NaPi-2-induced Na^+/P_i cotransport. If the coupling ratio of Na^+ versus P_i were 2:1, transport of divalent P_i would be electrically silent, contrasting with the high I_P measured at pH 7.8. Consequently, the coupling ratio of Na^+ to P_i must be greater than 2:1, resulting in electrogenic transport of both monovalent and divalent P_i. The discrepancy to a coupling ratio of 2:1 as derived from P_i-transport studies in brush border membrane vesicles may result from different factors—e.g., from a heterogeneity/multiplicity of Na^+/P_i cotransporters in proximal tubule (16, 17)—as well as from differences in transport analysis—i.e., tracer studies under non-voltage-clamp conditions versus short-circuit current measurements in oocytes.

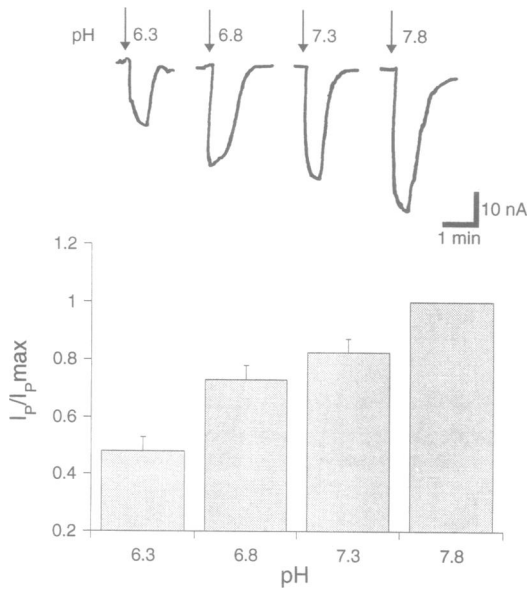

FIG. 5. I_P as a function of pH. (*Lower*) Correlation between I_P and pH (arithmetic means ± SEM, $n = 4$). I_P was normalized against maximal I_P ($I_P/I_{P(max)}$), which was always obtained at pH 7.8. (*Upper*) Characteristic corresponding tracings of I_P at a holding potential of −50 mV. Arrows indicate the starts of 30-sec period of superfusion with 1 mM P_i.

Conclusions. The present observations demonstrate electrogenic, Na^+-dependent, and pH-sensitive P_i transport by a cloned rat renal brush border membrane transporter (NaPi-2) expressed in *Xenopus* oocytes. Acidification reduces the transport of P_i, mainly by decreasing the Na^+ affinity of the carrier. The data also suggest transport of mono- as well as divalent P_i. The observations of NaPi-2-dependent inward I_P in *X. laevis* oocytes parallel those of proximal tubular and brush border membrane P_i transport. Since P_i transport under non-voltage-clamp conditions is decreased by depolarization, the inhibition of proximal tubular P_i transport by other Na^+-coupled transport can be explained by a decrease in the driving force for Na^+/P_i cotransport (i.e., the electrochemical potential difference for Na^+).

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