INORGANIC PHOSPHATE (P_i) is vital to all cells and organisms for both their structural and metabolic needs. As most cells maintain an inside-negative membrane potential, the anionic P_i cannot accumulate in the cytosol by passive diffusion. In epithelial cells, regulated P_i uptake is principally mediated by type II sodium-phosphate cotransporters (NaPi-II), which drive uphill transport of P_i by coupling it to the Na\(^{+}\) type II sodium-phosphate cotransporters (NaPi-II), which drive uphill transport of P_i by coupling it to the Na\(^{+}\) type II sodium-phosphate cotransporters (NaPi-II). The related type IIb isoform (for a recent review, see Ref. 28).

The cloning of cDNAs for NaPi-II cotransporters has enabled the study of their transport mechanism in more detail by exogenous expression, notably in Xenopus laevis oocytes. Work focusing mainly on the renal type IIa isoform has established that P_i transport is electrogenic and that transport involves the ordered binding and translocation of three Na\(^{+}\), one P_i, and one net positive charge per transport cycle (for reviews, see Refs. 8 and 10). The most detailed kinetic analysis has been carried out on rat and flounder NaPi-II isoforms, where pre-steady-state current analysis was employed to show that Na\(^{+}\) binds first, followed by P_i and two more Na\(^{+}\) ions (7, 12). Translocation of the fully loaded carrier is electroneutral, with electrogenicity arising from the return of the empty carrier to its initial conformation in an electrogenic step. In addition to cotransport, in the absence of P_i, NaPi-II mediates a Na\(^{+}\)-dependent leak current that is blocked by phosphonoformic acid (PFA), a competitive inhibitor of P_i transport. P_i transport by NaPi-II decreases with decreasing pH, in part because of titration of the preferred transported substrate (HPO_4^{-2}) (11) and in part because protons are able to interact with H\(^{+}\)-dependent partial reactions in the transport cycle (9).

Understanding the kinetics of P_i transport by NaPi-II is complicated because of the number of interdependent parameters involved. The rate of transport is influenced by the availability of Na\(^{+}\) and P_i, by pH and by membrane voltage. Furthermore, alterations in any one of these parameters affect the interactions of the others with the transport cycle. In this work, we have characterized the complex interrelationship of substrate concentrations, pH, and membrane voltage in human NaPi-II by analyzing the dependency of steady-state and pre-steady-state currents on these parameters. This has allowed us to identify additional partial reactions in the transport cycle that are influenced by pH and/or voltage.

MATERIALS AND METHODS

Molecular Biology and Oocyte Expression

The cDNA encoding for human NaPi-II was subcloned into a KSM expression vector to improve its expression in X. laevis oocytes, as described previously (36). The plasmid was linearized using XbaI (Promega) and used as a template for the synthesis of capped cRNA using a Message Machine T3 kit (Ambion).

Virkki, Leila V., Ian C. Forster, Jürg Biber, and Heini Murer. Substrate interactions in the human type IIa sodium-phosphate cotransporter (NaPi-IIa). Am J Physiol Renal Physiol 288: F969–F981, 2005. First published December 21, 2004; doi:10.1152/ajprenal.00293.2004.—We have characterized the kinetics of substrate transport in the renal type IIa human sodium-phosphate cotransporter (NaPi-IIa). The transporter was expressed in Xenopus laevis oocytes, and steady-state and pre-steady-state currents and substrate uptakes were characterized by voltage-clamp and isotope flux. First, by measuring simultaneous uptake of a substrate ($^{32}$P_i, $^{22}$Na) and charge in voltage-clamped oocytes, we established that the human NaPi-IIa isoform operates with a Na/P_i charge stoichiometry of 3:1:1 and that the preferred transported P_i species is HPO_4^{-2}. We then probed the complex interrelationship of substrates, pH, and voltage in the NaPi-IIa transport cycle by analyzing both steady-state and pre-steady-state currents. Steady-state current measurements show that the apparent HPO_4^{-2} affinity is voltage dependent and that this voltage dependence is abrogated by lowering the pH or the Na\(^{+}\) concentration. In contrast, the voltage dependency of the apparent Na\(^{+}\) affinity increased when pH was lowered. Pre-steady-state current analysis shows that Na\(^{+}\) ions bind first and influence the preferred orientation of the transporter in the absence of P_i. Pre-steady-state charge movement was partially suppressed by complete removal of Na\(^{+}\) from the bath, by reducing extracellular pH (both in the presence and absence of Na\(^{+}\)), or by adding P_i (in the presence of 100 mM Na\(^{+}\)). None of these conditions suppressed charge movement completely. The results allowed us to modify previous models for the transport cycle of NaPi-IIa by including voltage dependency of HPO_4^{-2} binding and proton modulation of the first Na\(^{+}\) binding step.

Xenopus laevis oocyte; electrophysiology; two-electrode voltage clamp; stoichiometry; structure-function
Stage V-VI defolliculated oocytes from *X. laevis* were isolated and maintained as described previously (40). Oocytes were injected with 50 nl of cRNA (0.2 μg/μl) encoding NaPi-IIa. Control oocytes were injected with 50 nl of water. Oocytes were incubated at 18°C in modified Barth’s solution containing (in mM) 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 NaHCO₃, 2 Ca(NO₃)₂, and 7.5 HEPES, pH 7.5, adjusted with TRIS. The solution was supplemented with 5 mg/l doxycyclin. Electrophysiology and radiotracer flux experiments were performed 2–5 days after injection. Each data set was obtained from at least two batches of oocytes from two different donor frogs.

**Reagents**

All standard chemicals and reagents were obtained from either Sigma or Fluka Chemie. [³²P]orthophosphate and [²²Na] were purchased from New England Nuclear and Amersham, respectively.

### Two-Electrode Voltage Clamp and Radiotracer Uptake

**Simultaneous voltage clamp and [³²P]/[²²Na] uptake.** For simultaneous voltage-clamp and radiotracer uptake experiments, we used an OC-725C oocyte clamp (Warner Instruments) connected to data-acquisition hardware (Digidata 1322A, Axon Instruments). A computer running pClamp8 (Axon Instruments) was used to control the clamp and record currents. The oocyte was placed in a superfusion chamber (based on a design by D. D. F. Loo, UCLA) with an effective volume of 24 μl and superfused at a rate of 100 μl/min using a peristaltic pump, with excess solution removed by a suction pump. The oocyte was initially voltage clamped to −50 mV and superfused with ND-100 solution (in mM: 100 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES, titrated to pH 7.4 using TRIS) until a stable baseline was reached. The superfusate was then switched to a solution containing 40 mM Na⁺ (ND-40, Na⁺ replaced by choline). The oocyte was exposed for 10–15 min to ND-40 solution containing 2 mM cold Pi (added as a mixture of K₂HPO₄/KH₂PO₄ of the appropriate pH) and [³²P]orthophosphate (specific activity 3,000–4,000 dpm/nmol Pi) and [²²Na] (specific activity 1,000 dpm/nmol Na). After a 10- to 15-minute period, the perfusate was switched back to ND-40. Both application and washout of Pi were performed 2–5 days after injection. Each data set was obtained from at least two batches of oocytes from two different donor frogs.

The net charge Q translocated by NaPi-IIa was calculated from current tracings by first subtracting the baseline holding current and then integrating the area under Pi-induced current curves using the Clampfit module in pClamp. The Na⁺ and Pi uptake for each oocyte were then correlated to Q using linear regression analysis.

**Two-electrode voltage clamp: kinetic characterization.** We used a custom-built voltage clamp optimized for fast clamping speed (7) to make recordings from control oocytes and oocytes expressing NaPi-IIa. The voltage clamp was controlled and data were acquired using a computer running pClamp 8 software (Axon Instruments), which also controlled the valves for solution switching. Solutions were cooled to 20–22°C before introduction to the oocyte-recording chamber at a rate of 5 ml/min. The oocyte was initially superfused with ND-100 solution, pH 7.4. Different concentrations of Pi were obtained by adding K₂HPO₄/KH₂PO₄, the proportions of which were adjusted to give the desired pH. PFA was first dissolved in water before addition to the desired solution. In some experiments, it was necessary to substitute CaCl₂ with BaCl₂ to suppress the activation of endogenous Cl⁻ channels at hyperpolarizing voltages. In solutions where the Na⁺ concentration was varied (ND-0 to ND-100), NaCl was substituted equimolarly with choline Cl⁻.

**Measurement of apparent Pi and Na⁺ affinities.** The oocyte was initially perfused with ND-100 solution, and the membrane potential was clamped to −50 mV. The holding current was recorded continuously. To measure Pi-induced currents, the superfusate was switched to one containing Pi, and deflection in the holding current was monitored. When the current had reached its maximum, the perfusate was switched back and washout of Pi was monitored by observing the return of the holding current to baseline. When Pi-induced currents were to be recorded for another Na⁺ concentration or pH, the holding current was first allowed to stabilize in the new baseline solution before the switch to one including Pi.

For determining apparent *Kₖ* for Pi (*Kₖₚ*), Pi-induced current deflections were measured using different Pi concentrations while the Na⁺ concentration was kept constant. For determining the apparent *Kₖ* for Na (*Kₖₙ*), the oocyte was first perfused with a specific concentration of Na⁺ before the switch to a solution containing Pi, and the same Na⁺ concentration. To enable comparison between different oocytes and to monitor current rundown, Pi-induced currents were recorded using a standard solution (ND-100, 1 mM Pi, pH 7.4) at the beginning and at the very end of each experiment. Pi-induced currents recorded in other conditions were then normalized to the current obtained with the standard solution.

The apparent *Kₖₚ* and *Kₖₙ* were determined by fitting data with the modified Hill equation

\[
I_{\text{Pi}} = I_{\text{PFA-inhibitable}} \left( \frac{[S]}{[S]^n + (K_{\text{Pi}})^n} \right) \]

where *Iₚ* is the Pi-induced current, [S] is the concentration of the substrate (Na⁺ or Pi), *Kₚ* is the concentration of the substrate that gives a half-maximum response, and *n* is the Hill coefficient. For determination of *Kₚ*, *n* was constrained to 1.

The voltage dependencies of *Kₚ* and *Kₙ* were measured by applying holding potentials from −160 to +40 mV and recording currents in the presence and absence of PFA, as described previously (7). Currents recorded in the absence of Pi were subtracted from those recorded in the presence of Pi to obtain *Iₚ*. The amount of leak current mediated by NaPi-IIa in ND-100 was estimated by subtracting Pi with 1 mM PFA, a competitive blocker of NaPi-IIa (17). To account for the differences in expression levels between individual oocytes, the data obtained from each oocyte were normalized to *Iₚ* recorded at −100 mV with 100 mM Na⁺ and 1 mM Pi, in the bath at pH 7.4. *Kₚ* and *Kₙ* were determined by fitting the data at each voltage with the modified Hill equation plus a variable offset.¹

¹ It is necessary to include an offset in the equation, because an error is introduced in the estimate of *Iₚ* when current recordings obtained in the presence and absence of Pi are subtracted. This occurs because in the absence of NaPi-IIa operates in a Na⁺-independent leak mode. Because the leak current is suppressed by Pi (17, 19), recordings made in the absence of Pi contain more leak current than recordings made in the presence of Pi. The amount of leak current present in each experiment is hard to define, since it is a function of both Na⁺ and Pi concentrations. Furthermore, although the current is fully suppressed at 1 mM Pi and 100 mM Na⁺ (7, 19), the *Kₙ* for the suppression of the leak current is not known. PFA is a blocker of NaPi-II and fully suppresses the leak current at 1 mM (and 100 mM Na⁺) as well as provides an estimate for the total amount of leak. However, its *Kₙ* for blocking the leak current is not known. In the human NaPi-IIa isoform, the leak current, measured as the PFA-inhibitable component, has a reversal potential around −25 mV and contributes little to the total current measured at −50 mV (see Fig. 4A). However, at more extreme potentials, the error introduced by the subtraction procedure becomes larger.
Pre-steady-state charge movement. Pre-steady-state current relaxations were acquired using voltage steps from a holding potential of −60 mV to test voltages in the range −160 to +80 mV. Signals were sampled at 50-μs intervals, low-pass filtered at 500 Hz, and four current tracings were averaged for the final recording. To improve signal-to-noise ratios, capacitive current compensation was used to partially suppress the capacitive transient and thereby allow recordings to be made at a high gain without amplifier clipping distorting the acquired signal. Relaxations were quantified by fitting a decaying double exponential function using a fitting algorithm based on the Chebychev transform supplied with pClamp. The fast component (τ ~ 0.7 ms) was assumed to represent endogenous charging of the oocyte membrane, as it showed little voltage dependence. The exogenous charge movement \( Q \) at each voltage was obtained by numerically integrating the slower component, extrapolated to the step onset. For some oocytes, it was necessary to apply a linear baseline correction to eliminate contamination from endogenous currents activated at extreme depolarizing or hyperpolarizing potentials.

The total charge \( Q \) moved per transport cycle was estimated by fitting the charge-voltage (\( Q-V \)) data with a two-state Boltzmann equation of the form

\[
Q = Q_{\text{hyp}} + Q_{\text{max}} \left[ 1 + \exp\left( -ze(V - V_{0.5})(kT)\right) \right] 
\]

where \( Q_{\text{max}} \) is the total charge available for translocation, \( Q_{\text{hyp}} \) is the charge translocated at the hyperpolarizing limit, \( V_{0.5} \) is the voltage at which the charge is distributed equally between two states, \( z \) is the apparent valence (a product of the valency of the charge and the fraction of the transmembrane field sensed by that charge), \( e \) is the elementary charge, \( k \) is Boltzmann’s constant, and \( T \) is the absolute temperature.

Statistical Analysis and Curve Fitting

Statistical analysis of the data was carried out using a two-tailed t-test or one-tailed ANOVA with Tukey’s posttest, using GraphPad Prism 3.0 software (GraphPad Software). Data were fit with the fitting algorithms supplied with Prism 3.0 software using the appropriate equation as indicated in the text.

RESULTS

Voltage Clamp and Dual Uptake of Na\(^{+}\) and Pi

To determine the stoichiometry of human NaPi-IIa, we performed simultaneous measurements of Na\(^{+}\), Pi, and charge transfer in oocytes expressing human NaPi-IIa. In addition, we identified the preferred transported Pi species by performing the assay at two different pH values (the \( pK_a \) for H\(_2\)PO\(_4^-\)/HPO\(_4^{2-}\) is 6.8). Figure 1A shows a representative current trace for a NaPi-IIa-expressing oocyte voltage clamped to −50 mV in ND-40 solution at pH 7.5. At the indicated time, 2 mM Pi was added, together with radioactive \(^{22}\)Na and \(^{32}\)Pi. Application of Pi induces an inward current, and by integrating the area under the curve, we obtained the total amount of charge moved during the application of Pi. The amount of Pi and Na\(^{+}\) taken up by the oocyte during this time period was calculated from accumulated radioactivity in each individual cell. The Na\(^{+}\) and Pi uptake data were plotted as a function of the charge in Fig. 1, B (pH 7.5) and C (pH 6.8). Linear regression analyses showed that for both pH values tested, the ratio of Na\(^{+}\) to charge (3:1) and Pi to charge (1:1) remained constant. Also, when we plotted Na\(^{+}\) as a function of Pi, the ratio to Na to Pi was constant (3:1, not shown).

The purpose of performing the experiment at two different pH values was to determine the contribution of monovalent (H\(_2\)PO\(_4^-\)) and divalent (HPO\(_4^{2-}\)) Pi to the movement of charge. At pH 7.5, the ratio of divalent to monovalent Pi is 5:1, whereas at pH 6.8 monovalent and divalent forms are present in equal amounts. If both species were transported with similar efficien-

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**Fig. 1.** Simultaneous measurement of Pi, Na\(^{+}\), and charge movement. A: representative current tracing for an oocyte expressing type II human Na-Pi cotransporter (NaPi-IIa). At the onset of the trace, the oocyte is perfused with ND-40 solution, pH 7.5. Radioisotopes (\(^{22}\)Na, \(^{32}\)Pi) were applied simultaneously with 2 mM cold Pi, as indicated. For water-injected (control) oocytes, no deflection in the holding current was observed when Pi was applied (not shown). B: relationship between Na\(^{+}\) and Pi uptake and charge transfer for individual oocytes at pH 7.5. C: relationship between Na\(^{+}\) and Pi uptake and charge transfer for individual oocytes at pH 6.8. In B and C, the charge \( Q \) is obtained by integration of the Pi-induced current in traces similar to in A and expressed as charge equivalents (1 mol = 96,485.31 C). Na\(^{+}\) and Pi uptakes are calculated from the amount of isotope accumulated in each oocyte and the specific activity of isotope in the bath. Lines are linear regression fits to the data for the NaPi-IIa-expressing oocytes. Slopes Na\(^{+}\)-Q and Pi-Q are indicated in the graphs. The lines were forced through the mean values for Na/Pi uptake obtained in control oocytes. Each point represents data obtained from a single oocyte.
Fig. 2. Effect of pH on the Na+ dependence of Pi-induced current (I_Pi). A: representative current tracing for an oocyte expressing NaPi-IIa. The oocyte is perfused with solution containing different amounts of Na+ at pH 7.4 as indicated. At the indicated times, 1 mM Pi is applied to the oocyte. I_Pi is measured from the magnitude of the Pi-induced deflection in the holding current (indicated by dotted lines). Note that the baseline holding current depends on the Na+ concentration. B: I_Pi plotted as a function of the Na+ concentration. Data were acquired at 2 different pHs in the bath and with 3 different Pi concentrations (in mM). Data were fitted with the Michaelis-Menten equation (Eq. 1). Data for the fitted parameters are given in Table 1. Values are means ± SE; n = 4–6.

Effect of pH on Na+ Transport Kinetics in NaPi-IIa

The effect of pH on apparent K_m is first investigated using a constant holding potential of −50 mV. At this potential, contamination of the leak pathway to the Pi-dependent current is very small; i.e., the Pi-dependent current equals cotransporter current. The protocol is also less challenging for the oocyte and allows a more reliable estimate of I_Pi,max to be made, compared with the voltage-jump protocol used below.

We measured Pi-activated currents by continuously recording the holding current at −50 mV and monitoring Pi-induced downward deflection (Fig. 2A). The Pi-induced current (I_Pi) was plotted as a function of Na+ concentration and fitted with Eq. 1 to obtain apparent K_m, Hill coefficient n, and I_Pi,max (Fig. 2B). Experiments were carried out at pH 7.4 and 6.6. I_Pi was applied at 1 or 0.1 mM concentration for pH 7.4, and at 1 or 2 mM concentration for pH 6.6 (at pH 6.6, the HPO_4^{2−} concentration at 2 mM Pi is equal to the HPO_4^{2−} concentration at 1 mM Pi at pH 7.4). For each oocyte, I_Pi was also measured at pH 7.4, 1 mM Pi, and all data were normalized to this value for each individual oocyte.

Reducing pH from 7.4 to 6.6 at either a fixed total Pi or total HPO_4^{2−} concentration resulted in a large increase in K_m, as did reducing the total Pi from 1 to 0.1 (see Table 1). The Hill coefficient and I_Pi,max were less sensitive to pH; however, lowering the pH at a fixed total Pi of 1 mM caused a statistically significant increase in the Hill coefficient as well as a decrease in I_Pi,max. Thus K_m is highly sensitive to both pH and the concentration of the substrate HPO_4^{2−}, whereas the Hill coefficient and I_Pi,max are less so.

Effect of pH on Pi Transport Kinetics in NaPi-IIa

Next, we characterized how pH affects the apparent affinity for Pi by studying the kinetics of I_Pi over a range of pH values. Experiments were carried out as above using a constant holding potential of −50 mV and by applying Pi at various concentrations (Fig. 3). In addition, we acquired a data set for pH 7.4 with 50 mM Na+. For each oocyte, I_Pi obtained for each condition was normalized to that measured at ND-100, 1 mM Pi, pH 7.4.

I_Pi was plotted as a function of the concentration of divalent HPO_4^{2−} (Fig. 3B). Data were fitted with Eq. 1, and the calculated apparent affinity for HPO_4^{2−} (K_m Pi) and I_Pi,max was plotted as a function of pH in Fig. 3C. I_Pi,max was reduced, and K_m Pi increased, by reduced pH. Thus reducing pH affects both apparent K_m Pi and I_Pi,max. Note that if the affinity for phosphate was calculated as a function of the total Pi concentration (as K_m) the increase in K_m would be much larger for a given reduction in pH, because the concentration of the transported substrate (HPO_4^{2−}) is pH dependent (pK_a ∼6.8). However, the calculated I_Pi,max would be the same in both cases. Fitting I_Pi,max data in Fig. 3C with Eq. 1 gave an apparent inhibition constant (K_i) for H^+ of 400 ± 40 mM (corresponding to a pH of 6.4) and a Hill coefficient of −2.1 ± 0.6.

When we compare the effect of pH on the two cosubstrates Na+ and HPO_4^{2−}, we see that, for a reduction in pH from 7.4 to 6.6, K_m Pi was increased by 50% and I_Pi,max was reduced by 25%. For Na+ as the variable substrate, we find in Fig. 2 and Table 1 that K_m was increased by 57% and I_Pi,max was reduced by 15% for the same decrease in pH. Thus pH affects the kinetic parameters of steady-state transport of both substrates in a similar manner. Note that reducing Na+ from 100 to 50 mM causes a fourfold increase in K_m Pi, a

<table>
<thead>
<tr>
<th>pH</th>
<th>K_m (mM)</th>
<th>n</th>
<th>I_Pi,max (norm)</th>
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</thead>
<tbody>
<tr>
<td>7.4</td>
<td>1 mM Pi</td>
<td>40±2</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td></td>
<td>0.1 mM Pi</td>
<td>105±28*</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>6.6</td>
<td>2 mM Pi</td>
<td>71±8*</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td></td>
<td>1 mM Pi</td>
<td>82±8*</td>
<td>2.8±0.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, Hill coefficient. I_Pi,max, maximal Pi-induced current. Parameters were obtained by fitting the modified Hill equation (Eq. 1) to data in Fig. 2B. *Significantly different from the value obtained for 1 mM Pi, pH 7.4 (ANOVA) (P < 0.05).

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significantly larger effect than the twofold increase in $K_m^\text{HPO}_4^2$ seen with the maximal reduction of pH to 6.4. The opposite effect is seen for $I_{P1\text{max}}$, where lowering Na from 100 to 50 mM has a much smaller effect than lowering pH.

Voltage Dependency of $K_m^Pi$

Figure 4A shows $I_{P1}$ (filled symbols) and current blocked by PFA (○) as a function of the holding voltage in oocytes expressing NaPi-IIa. The PFA-blocked current represents current mediated by the leak pathway that operates in the absence of Pi. The true Na-Pi cotransport current (not shown) mediated by NaPi-IIa is therefore the sum of Pi-induced and leak currents, because the latter current is subtracted from current recordings made in the presence of Pi. (note that for nonsaturating Pi concentrations, some leak current will remain in the recordings made in the presence of Pi, which will reduce the error associated with the subtraction procedure). It can be seen (Fig. 4, A–C) that as the Pi (HPO$_4^2$) concentration is reduced, the slope of the current-voltage (I-V) relationship shallows and is ultimately inverted for the lowest HPO$_4^2$ concentration (0.008 mM). This is a consequence of the leak current and the subtraction procedure used to obtain the Pi-dependent current. Since the affinity of Pi for blocking the leak pathway is not known, estimating how much leak is left in the recordings made with a lower Pi concentration is not feasible. We have therefore chosen not to correct the data for leak.

The voltage dependency of the Pi-dependent current was also measured at pH 6.6 (Fig. 4B) and 50 mM Na, pH 7.4 (Fig. 4C). All data were normalized for each cell to the Pi-dependent current obtained at $-100 \text{ mV}$ with 100 mM Na, 1 mM Pi, and pH 7.4 (● in B and C). The data in Fig. 4, B and C, show that Pi-induced currents were reduced at all Pi concentrations, compared with the data in Fig. 4A. The flattening of the I-V curves at hyperpolarizing potentials, seen, in particular, for 50 mM Na$^+$ (Fig. 4C), indicates that the transport rate is determined by voltage-independent, rate-limiting steps under these conditions.

$K_m^\text{HPO}_4^2$ was calculated from data in Fig. 4, A–C, using Eq. 1 (we assume that only divalent HPO$_4^2$ is transported; Fig. 1), and plotted as a function of the holding potential in Fig. 4D. At 100 mM Na$^+$ and pH 7.4, $K_m^\text{HPO}_4^2$ is voltage dependent and varies inversely with voltage. This voltage dependency is reduced when the pH is lowered to 6.6. The voltage dependency of $K_m^\text{HPO}_4^2$ in ND-50, pH 7.4, also appears to be reduced; however, the error in estimating the parameter was exacerbated by the reduced signal-to-noise ratio at 50 mM Na$^+$. Reducing Na$^+$ significantly increased $K_m^\text{HPO}_4^2$ throughout the whole voltage range measured.

The voltage dependency of $I_{P1\text{max}}$ was extracted using Eq. 2 and plotted in Fig. 4E. Reducing pH from 7.4 to 6.6, or reducing the Na$^+$ concentration from 100 to 50 mM, caused a reduction in $I_{P1\text{max}}$ that became more pronounced at hyperpolarizing voltages. Interestingly, this decrease in $I_{P1\text{max}}$ was very similar in both conditions, although reducing the Na$^+$ concentration had a much stronger effect on $K_m^\text{HPO}_4^2$ than lowering pH.

Voltage Dependency of $K_m^{Na^+}$

Figure 5A shows the voltage dependency of the current induced by 1 mM Pi (0.8 mM HPO$_4^2$) at variable Na$^+$ concentrations and pH 7.4 for oocytes expressing NaPi-IIa. Reducing the Na$^+$ concentration also reduced the voltage dependency of $I_{P1}$, so that at 10 mM Na$^+$ the I-V curve was essentially flat. When pH was reduced to 6.6 while total Pi concentration was maintained at 1 mM (Fig. 5B), $I_{P1}$ decreased for all Na$^+$ concentrations tested. At low Na$^+$, the I-V curves acquired a negative slope due to uncompensated leak current (see above).

Data for each voltage were plotted as a function of Na$^+$ concentration, and $K_m^{Na^+}$ was calculated using Eq. 1 with a variable offset. Figure 5C shows $K_m^{Na^+}$ measured at pH 7.4 and 6.6 as a function of the holding potential. No voltage depen-
dency is seen for voltages more positive than $-80$ mV. However, below $-80$ mV, $K_m^{Na}$ increased with hyperpolarization. At pH 6.6, $K_m^{Na}$ also became voltage dependent at more positive voltages and was voltage independent only for potentials above 0 mV.

Figure 5D shows $I_{Pi, \text{max}}$ at pH 7.4 and 6.6 plotted as a function of voltage. There is an apparent reduction in $I_{Pi, \text{max}}$ at pH 6.6 compared with 7.4 throughout the voltage range tested. This result should be interpreted with caution, however, as the lack of saturation in Fig. 5B precludes a sufficiently reliable fit of the data to Eq. 1. We were unable to obtain reliable data using the voltage-jump protocol on oocytes using solutions with Na$^+$ concentrations above 125 mM.

Pre-Steady-State Kinetics: Na$^+$ Dependence of Charge Movement

To identify partial reactions in the NaPi-IIa transport cycle that are influenced by substrate and pH, we performed pre-steady-state current analysis. First, we examined the Na$^+$ dependence of pre-steady-state currents by exposing an oocyte to solutions with different Na$^+$ concentrations. Figure 6A shows a representative recording of currents acquired using the voltage-jump protocol in an oocyte expressing NaPi-IIa bathed in ND-100 solution at pH 7.4. After baseline correction and rescaling (Fig. 6B, left), pre-steady-state relaxations were clearly observed, superimposed on the oocyte capacitive charging. These relaxations were partially suppressed in the presence of Na$^+$.
ence of 1 mM Pi (Fig. 6B, middle) and were absent in control oocytes (Fig. 6B, right).

The charge $Q$ moved from $-60$ mV to each target potential was extracted (see MATERIALS AND METHODS), plotted as a function of target potential and fitted with Eq. 2. Figure 6C shows a $Q$-$V$ plot for oocytes superfused with Na$^+$ concentrations from 0 to 125 mM, and 100 mM Na$^+$ and 1 mM Pi$^-$ The parameters obtained from the fits are shown in Table 2. The maximum amount of charge available ($Q_{\text{max}}$) was suppressed when all Na$^+$ was removed from the superfusate. Maximum suppression of charge movement was observed when 1 mM Pi$^-$ was added to the superfusate containing 100 mM Na$^+$; however, none of the conditions used in Table 2 were able to fully suppress pre-steady-state currents. Quantitatively similar results were obtained when we extracted the charge moved from each test potential back to $-60$ mV (not shown), which confirmed charge balance.

The midpoint voltage $V_{0.5}$ was shifted in the negative direction when the Na$^+$ concentration was reduced, from $-8 \pm 12$ mV at 125 mM Na$^+$ to $-77 \pm 7$ mV at 10 mM Na$^+$ (Fig. 6D, Table 2). Removing all Na$^+$ in the bath caused no further change in $V_{0.5}$. Plotting $V_{0.5}$ as a function of log[Na$^+$] revealed a near-linear relationship for Na$^+$ concentrations $\geq 25$ mM, with a slope of $94 \pm 6$ mV/log[Na$^+$] (Fig. 6D, inset). The apparent valency ($z$) reported by the fit was between 0.40 and 0.52 for all the conditions tested, but the differences were not statistically significant.

**Pre-Steady-State Kinetics: Effect of pH**

To identify pH-sensitive steps in the transport cycle of NaPi-IIa, we examined the effect of lowering pH on pre-steady-state charge movement. $Q$-$V$ data were acquired in the presence and absence of Na$^+$, with and without 1 mM Pi$^-$. The $Q$-$V$ data shown in Fig. 7 were fitted with Eq. 2, and the parameters obtained from the fit are shown in Table 3. Lowering pH from 7.4 to 6.6 in ND-100 suppressed $Q_{\text{max}}$ and shifted $V_{0.5}$ from $-30 \pm 2$ to $-23 \pm 9$ mV. After the addition of 1 mM Pi$^-$ in either pH, $Q_{\text{max}}$ was suppressed to the same level in both cases ($\sim 40\%$). In the absence of external Na$^+$, a condition that already suppresses $Q_{\text{max}}$, reducing pH from 7.4 to 6.6 resulted in an additional but weak suppression of $Q_{\text{max}}$ ($\sim 10\%$) and a shift in $V_{0.5}$ from $-62 \pm 9$ to $-52 \pm 6$ mV. Including 1 mM Pi$^-$ in the bath in ND-0 solution did not cause any changes in the measured parameters, indicating that Pi$^-$ is only able to alter charge movement in the presence of external Na$^+$.

We also attempted to resolve pre-steady-state charge movements using the voltage protocol at pH 5.0 (not shown). At this pH, the holding current of the oocyte was unstable for voltage steps below $-140$ mV and above $+40$ mV, precluding further analysis. However, inspection of the current transients (acquired in either the presence or absence of Na$^+$) revealed that no pre-steady-state charge movement was visible and the transients looked like those obtained from water-injected (control) oocytes.
to the voltage-jump protocol. The tracing shows the full current response for voltage steps between −100 and +80 mV from an initial holding level of −60 mV. B: tracings were baseline adjusted and expanded to show the pre-steady-state currents after the step to the test potentials. Tracings are shown for the same oocyte as in A for 100 mM Na⁺ (left) and 100 mM Na⁺ + 1 mM P₁ in the bath (middle) on the same scale. The right tracing shows the pre-steady-state current response for a water-injected (control) oocyte. C: steady-state charge distribution (Q-V) determined at different concentrations of Na⁺ and with 100 mM Na⁺ + 1 mM P₁. Data were normalized to the maximum amount of movable charge, which was observed with 100 mM Na⁺ in the bath, and the baseline was shifted so that all curves superimpose at the upper limit. Continuous lines are fits to the Boltzmann equation (Eq. 2). Parameters for the fits are given in Table 2. D: plot of the estimated midpoint voltage (V₀.₅) of Q-V (obtained from fitting the data in C) as a function of the Na⁺ concentration. Inset: V₀.₅ plotted against log[Na⁺]. The slope of the linear regression line passing through the linear portion of this relationship was 95 ± 6 mV/log[Na⁺]. Based on a simple 2-state model for Na⁺ interaction, this would suggest that a single Na⁺ ion moves 60% of the transmembrane field to its binding site. Values are means ± SE; n = 5–6.

DISCUSSION

Stoichiometry of hNaPi-IIa

Determining the stoichiometry of electrogenic transporters is often accomplished by simultaneously measuring the transfer of charge and substrate (e.g., Refs. 3, 4, 21, and 33). Substrate transport is measured either as the flux of a radioactive isotope (unidirectional transport) or from changes in substrate concentration (net transport). Another approach, based on thermodynamics, is to measure changes in the current reversal potential (Eᵣₑᵥₑ) in response to changes in substrate concentration (2, 37); however, this approach requires that Eᵣₑᵥₑ can be measured with the voltage window usable for the preparation, and for many Na⁺-coupled transporters this is not often possible. Also, this approach is compromised if the substrate induces significant nonstoichiometric current, as for neurotransmitter transporters (5, 13, 24, 34). Using a kinetic approach, the Hill coefficient is often used to infer stoichiometry. However, since the Hill coefficient is a measure of cooperativity of substrate binding, it can only provide a lower estimate of the number of binding sites (see Ref. 39, for example).

As we were unable to record current reversal potentials in oocytes expressing NaPi-IIa (for a 3:1 Na:Pi stoichiometry, the expected Eᵣₑᵥₑ exceeds +150 mV), we measured simultaneous uptake of Na⁺, P₁, and charge. The results show a Na⁺:charge ratio of 3:1, P₁:charge ratio of 1:1, and a Na⁺:P₁ ratio of 3:1 for both pH 7.5 and 6.8. From these data, we conclude that the transport cycle of human NaPi-IIa involves the tranlocation of 1 HPO₄⁻, 3 Na⁺, and 1 positive net charge. This result is in full agreement with previous studies of the rat and flounder isofoms (11).

Table 2. Na dependency of pre-steady-state charge movement

<table>
<thead>
<tr>
<th>[Na] mM</th>
<th>Qmax (norm)</th>
<th>χ</th>
<th>V₀.₅ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.68±0.07</td>
<td>0.50±0.07</td>
<td>−74.7±2*</td>
</tr>
<tr>
<td>10</td>
<td>0.89±0.1</td>
<td>0.40±0.05</td>
<td>−77.7±2*</td>
</tr>
<tr>
<td>25</td>
<td>0.97±0.09</td>
<td>0.43±0.05</td>
<td>−76.2±2*</td>
</tr>
<tr>
<td>50</td>
<td>1.0±0.1</td>
<td>0.45±0.06</td>
<td>−52.5±2*</td>
</tr>
<tr>
<td>75</td>
<td>0.92±0.09</td>
<td>0.48±0.07</td>
<td>−35.5±2*</td>
</tr>
<tr>
<td>100</td>
<td>1.0±0.06</td>
<td>0.52±0.05</td>
<td>−23.4</td>
</tr>
<tr>
<td>125</td>
<td>0.85±0.16</td>
<td>0.48±0.11</td>
<td>−8±12</td>
</tr>
<tr>
<td>100+P₁</td>
<td>0.44±0.12*</td>
<td>0.47±0.17</td>
<td>−90±20*</td>
</tr>
</tbody>
</table>

Values are means ± SE, [Na]. Na concentration; Qmax, maximal net charge; χ, apparent valence; V₀.₅, voltage at which the charge is distributed equally between 2 states. Parameters were obtained by fitting a Boltzmann function (Eq. 2) to data in Fig. 6C. *Significantly different from the value obtained for 100 mM Na⁺ (Qmax) or 125 mM Na⁺ (V₀.₅; ANOVA) (P < 0.05).
This finding contrasts with other studies, which concluded that both monovalent and divalent $P_i$ are transported by human NaPi-IIa heterologously expressed in oocytes (1, 26). Busch and co-workers (1) reported that decreasing pH from 7.8 to 6.8 at 150 mM $Na^+$ enhanced the $P_i$-induced current, which would result from transport of more monovalent $P_i$, and hence more charge, at acidic pH. We repeated these experiments, but in our hands reducing the pH from 7.8 to 6.8 at 150 mM $Na^+$ produced an ~10% reduction in the $P_i$-induced current (Virkki LV, unpublished observations). More recently, Moschen and co-workers (26) used oocytes expressing the human NaPi-IIa isoform to compare changes in intracellular pH (pHi) induced by $P_i$ at pH 6.0 and 8.0, concluding that both monovalent and divalent $P_i$ are transported. However, their data show that applying $P_i$ at pH 6.0 (monovalent/divalent ratio 6.3:1) induces significant alkalization of the oocyte cytosol (pHi ~7.3, monovalent/divalent ratio 1:1.6), which shows that the affinity of the transporter for divalent $P_i$ exceeds at least 20-fold its affinity for monovalent $P_i$. Their data thus support the conclusion that divalent $P_i$ is the preferred transported species, whereas transport of $H_2PO_4^-$ is negligible.

<table>
<thead>
<tr>
<th>pH</th>
<th>$Q_{max}$ (norm)</th>
<th>$z$</th>
<th>$V_{o.s.}$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>1.0 ± 0.03</td>
<td>0.59 ± 0.03</td>
<td>-30 ± 2</td>
</tr>
<tr>
<td>6.6</td>
<td>0.60 ± 0.09</td>
<td>0.57 ± 0.14</td>
<td>-23 ± 9</td>
</tr>
<tr>
<td>7.4</td>
<td>0.41 ± 0.10</td>
<td>0.61 ± 0.23</td>
<td>-67 ± 14</td>
</tr>
<tr>
<td>6.6</td>
<td>0.43 ± 0.06</td>
<td>0.47 ± 0.10</td>
<td>-60 ± 9</td>
</tr>
<tr>
<td>0 mM $Na^+$</td>
<td>0.56 ± 0.08</td>
<td>0.55 ± 0.13</td>
<td>-62 ± 9</td>
</tr>
<tr>
<td>6.6</td>
<td>0.44 ± 0.05</td>
<td>0.51 ± 0.08</td>
<td>-52 ± 6</td>
</tr>
<tr>
<td>0 mM $Na^+$ + $P_i$</td>
<td>0.53 ± 0.08</td>
<td>0.56 ± 0.12</td>
<td>-67 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Parameters were obtained by fitting a Boltzmann function (Eq. 2) to the data in Fig. 7, A and B. Significantly, different from the value obtained at pH 7.4 for the same condition (ANOVA) ($P < 0.05$).

Substrate Interactions

The experiments in Figs. 3 and 4 show that with $P_i$ ($HPO_4^{2-}$) as the variable substrate, $K_mHPO_4^{2-}$ is increased and $I_{P_i, max}$ is decreased when the $Na^+$ concentration is reduced. A similar dependency of $K_{mP_i}^Na$ ($I_{P_i, max}$ was not determined) on Na concentration was observed for the mouse NaPi-IIa isoform (14), the flounder isoform (12), and the rat isoform (7). However, when $Na^+$ is the variable substrate, an increase in $K_{mP_i}^Na$ is observed with reduced $P_i$ concentrations, but in this case $I_{P_i, max}$ is minimally affected (Table 1). A similar effect was observed for the rat isoform (7). In contrast, Hartmann et al. (14) saw no effect of reducing $P_i$ on $K_{mNa}$ of the mouse isoform ($I_{P_i, max}$ was not determined). Given an apparent $K_{mP_i}^{Na}$ of ~0.07 mM at pH 7.4, this discrepancy might be explained by the smaller reduction in $P_i$ used by Hartmann et al. from 1 to 0.3 mM $P_i$, compared with this study (1 to 0.1 mM $P_i$).

The fact that NaPi-IIa cotransport involves three $Na^+$ ions per transport cycle complicates the interpretation of the results based on analysis of steady-state current only. Analysis of pre-steady-state charge movement on the flounder (12) and rat (7) NaPi-IIa isoforms indicates that at least one of the three $Na^+$ ions binds before $P_i$, as shown in the eight-state kinetic scheme presented in Fig. 8. The model is based on the results of this and previous studies (7, 9, 12). In this model, one $Na^+$ binds first, followed by one $HPO_4^{2-}$ and then two more $Na^+$...
Fig. 8. Modeling the transport cycle of human NaPi-IIa. A: kinetic scheme for the transport cycle of NaPi-IIa. Transitions that are influenced by voltage are identified by thick arrows: translocation of the empty carrier (1–8), first Na\(^+\) binding step (1–2), \(\text{HPO}_4^{2-}\) binding (2–3), and last Na\(^+\) debinding step (7–8). The leak pathway (2–7) operates in the absence of \(\text{HPO}_4^{2-}\). PFA is thought to bind to state 2 and place the system in a state that cannot perform cotransport or leak (2*). Protons interact with the first Na\(^+\) binding step (1–2), placing the carrier in an inhibited state (1*), and with the transition of the empty carrier (8–1). They may also interact with the binding of the last two Na\(^+\) ions (3–4), so that the protein favors occupancy of a state where cotransport of the substrate is no longer possible (3*). No information is available about the steps involving release of substrates to the internal side (hatched arrows), but they are depicted as mirror images of those taking place on the external side (transitions 5–6–7–8). B: simulated steady-state current-voltage relationships with variable \(\text{HPO}_4^{2-}\) and 100 mM Na. C: simulated steady-state current-voltage relationships with variable Na and 0.8 mM \(\text{HPO}_4^{2-}\). D: \(K_{\text{m,Na}}\) and \(K_{\text{m,PO}_4}\) generated by fitting the simulated data in B and C to Eq. 1. E: simulated steady-state Q-V for extracellular Na concentration ([Na]o) ranging between 125 and 0 mM. Data were fitted with Eq. 2 (solid lines). F: \(V_{\text{m,Na}}\) data from the fit of data in E with Eq. 2. The rate constants (in s\(^{-1}\)) used in all the simulations were as follows: \(k_{12} = 1.700[\text{Na}]_o\exp(-\alpha_1 eV/kT)\), \(k_{23} = 200\exp(\alpha_1 (1 + \delta) eV/kT)\), \(k_{24} = 1.600[\text{HPO}_4]_o\exp(\beta eV/kT)\), \(k_{34} = 500\exp(-\beta eV/kT)\), \(k_{43} = 40,000[\text{Na}]_o^2\), \(k_{44} = 200\), \(k_{45} = 1.000\), \(k_{54} = 25\), \(k_{55} = 100,000[\text{Na}]_o^2\), \(k_{67} = 100\), \(k_{56} = 20,000[\text{HPO}_4]_o\), \(k_{58} = 25\exp(-\alpha_2 eV/kT)\), \(k_{67} = 10,000[\text{Na}]_o\exp(\alpha_2 eV/kT)\), \(k_{58} = 40\exp(-\gamma eV/kT)\), \(k_{18} = 280\exp(\gamma (1 - \delta) eV/kT)\). The fractions of the electrical field that are sensed by the hypothetical charge moving through the membrane electrical field are represented by \(\alpha\), \(\beta\), and \(\gamma\) and were set as follows: \(\alpha_1 = 0.2\), \(\alpha_2 = 0.65\), \(\beta = 0.05\), and \(\gamma = 0.25\); \(\delta\) is an asymmetry factor that defines the position of the energy barrier within the transmembrane field and was set to 0.9; and [\(\text{Na}\)]o is intracellular concentration. To satisfy the condition for microreversibility, \(k_{56}\) was defined according to the relationship \(k_{56} = k_{56} \times k_{64} \times k_{43} \times k_{32} \times k_{21} \times k_{16} \times k_{56}/(k_{65} \times k_{54} \times k_{43} \times k_{32} \times k_{21} \times k_{16} \times k_{56}) = 2.574\). V = voltage, e = elementary charge, \(k = \) Boltzmann’s constant, and \(T = \) absolute temperature (293 K); [\(\text{Na}\)]o = 0–125 M, [\(\text{Na}\)]i = 0.01 M, \([\text{HPO}_4^{2-}]_o = 0–1\) mM, and \([\text{HPO}_4^{2-}]_i = 0.1\) mM, pH 7.4.
ions. The substrate-bound carrier then transitions to the internal side and returns empty after releasing the substrate.

The dependencies of $K_m$ and $I_{P_i \text{max}}$ on the cotransported substrate described above can be reconciled with the model presented in Fig. 8 if the first Na$^+$ binding site (transitions 1–2) has a higher affinity for Na$^+$ than the latter ones (transitions 3–4). In that case, the first Na$^+$ site is fully occupied at Na$^+$ concentrations where only a fraction of the latter Na$^+$ binding sites are occupied, and the system behaves (at saturating Na$^+$ concentrations) as if P$_i$ was the first substrate to bind.

Voltage Dependency of Steady-State Transport

Voltage dependencies of $K_m^{P_i}$ and $K_m^{Na}$ were previously examined in the rat NaPi-IIa isoform (7). At maximal Na$^+$ (100 mM), the apparent P$_i$ affinity of rat NaPi-IIa was weakly voltage dependent, with a decrease in $K_m^{P_i}$ seen at hyperpolarizing potentials. The effect was more pronounced at a lower Na$^+$ concentration (50 mM). This contrasts with the behavior seen for the human NaPi-IIa isoform, where $K_m^{P_i}$ ($K_m^{HPO_4^{2-}}$) increased markedly with hyperpolarization (Fig. 4). Lowering the Na$^+$ concentration and decreasing pH from 7.4 to 6.6 abrogated the voltage dependency. In contrast, the voltage dependency of $K_m^{Na}$ of human NaPi-IIa was increased when pH was lowered to 6.6 in the voltage range examined (Fig. 5), with an increase in $K_m^{Na}$ seen at hyperpolarizing potentials. Thus the voltage dependencies of $K_m^{P_i}$ and $K_m^{Na}$ of human NaPi-IIa are modulated in opposite ways by pH.

The finding of a voltage-dependent $K_m^{P_i}$ is novel for type II Na-Pi cotransporters. Previous models based on the rat NaPi-IIa isoform assume that P$_i$ binding is voltage independent (7, 12). The finding that at increasingly negative potentials the apparent affinity for negatively charged HPO$_4^{2-}$ is decreased suggests that at least in the human isoform, the P$_i$ binding site lies within the membrane electrical field.

Pre-Steady-State Charge Movement: Na$^+$ Dependency

Pre-steady-state currents have been analyzed for several different Na$^+$-coupled transporters, such as Na$^+$-dependent glucose (20, 29, 30), GABA (25), glutamate (38), iodine (4), and nucleoside (33) transporters, and are thought to arise from voltage-dependent conformational changes in the carrier and/or movement of ion(s) to binding site(s) within the membrane electric field. The pre-steady-state charge distribution is often approximated by a two-state Boltzmann function (15, 20, 23), yielding estimates of $Q_{max}$, $V_{0.5}$, and $z$. In the absence of a substrate, all Na$^+$-coupled transporters mentioned above show a negative shift in $V_{0.5}$ with reducing Na$^+$. In contrast, large differences between transporters are observed with respect to the Na$^+$ dependency of $Q_{max}$. For example, $Q_{max}$ was fully suppressed by removal of Na$^+$ in the GABA transporter mGAT3 (31), whereas $Q_{max}$ appears virtually independent of Na$^+$ in the iodine transporter NIS (4).

Analysis of Na$^+$ dependence of the pre-steady-state charge movement for human NaPi-IIa shows that $Q_{max}$ is relatively independent of Na$^+$ concentration and that significant charge movement was still observed in the complete absence of Na$^+$ (Table 2). This indicates that both the empty carrier (transitions 8–1 in Fig. 8) and the first Na$^+$ binding step (transitions 1–2 in Fig. 8) contribute to charge movement. Furthermore, a marked negative shift in $V_{0.5}$ is seen with reduced external Na$^+$ (Fig. 6D). For Na$^+$ concentrations of 25–125 mM, $V_{0.5}$ was a near-linear function of log[Na$^+$], with a slope of $\sim$100 mV per 10-fold change in the Na$^+$ concentration. This is similar to that seen for other Na$^+$-dependent transporters, such as SGLT1 (15) and GAT1 (25), and is reconcilable with a Nernstian dependency of $V_{0.5}$ on a single Na$^+$ that moves through a fraction of the transmembrane field to its binding site. According to the slope of the $V_{0.5}$ vs. log[Na$^+$] data (Fig. 6D, inset), the hypothetical Na$^+$ binding site would be located at an electrical distance corresponding to 60% of the transmembrane electrical field, relative to the external membrane face.\footnote{We assume a simple two-state model for the first Na$^+$ binding (states 1 and 2 in Fig. 8), from which the fraction $\alpha$ of the membrane electrical field sensed by the ion can be estimated. $V_{0.5}$ is the voltage at which the two states are equally occupied at each extracellular Na$^+$ concentration ($[Na_0]$), at which $k_{12} = k_{21}$, and the slope of the relationship $V_{0.5}/\ln[Na_0] = kT/2e$. However, because the empty carrier also contributes to charge movement, and its relative contribution is higher at low [Na$^+$], the method underestimates the slope, and hence overestimates $\alpha$. For a definition of symbols, see the legend to Fig. 8.}

When the Na$^+$ concentration was reduced to below 25 mM, the linearity was lost. This is most likely a consequence of fitting a single Boltzmann to the $Q-V$ data, although at least two voltage-dependent steps (binding of Na$^+$ and transition of the empty carrier) are expected to contribute charge movement. As the Na$^+$ concentration is changed, the relative contribution of these steps is altered, but the relative contribution of each remains unquantified. Unfortunately, it was not possible to fit the data to more than one Boltzmann function, a procedure that could have enabled us to separate the two components (18). Nevertheless, we can conclude that Na$^+$ ions affect the preferred orientation of the Na$^+$-bound carrier. As the external Na$^+$ concentration is raised, the transporter is increasingly assuming the outward-facing configuration, presumably ready for a P$_i$ (HPO$_4^{2-}$) ion to bind.

Applying P$_i$ (at 100 mM Na) significantly suppressed pre-steady-state charge movements (Table 2). However, in contrast to previous studies on rat (7) and flounder (12) isoforms, significant charge movement could still be observed in the human isoform in the presence of P$_i$. That also the P$_i$-bound carrier contributes to pre-steady-state charge movement is paralleled by the observation that the apparent affinity for P$_i$ binding in steady-state current measurement is voltage dependent, further supporting the idea that the P$_i$ (HPO$_4^{2-}$) binding site lies within the transmembrane electrical field.

Pre-Steady-State Charge Movement: Effect of pH

Examining the effect of reducing extracellular pH on pre-steady-state charge movement reveals that decreasing external pH from 7.4 to 6.6 markedly suppresses the maximum charge detected in the presence of 100 mM Na$^+$ (see Fig. 7 and Table 3). Also, in the absence of external Na$^+$ we observed suppression of charge movement by low pH. The results indicate that protons can interact with both the first Na$^+$ binding step and the transition of the empty carrier. Analysis of the pH dependency of the P$_i$-induced steady-state current supports the notion that protons are able to interact with at least two sites in the protein, as a Hill coefficient of $\sim$2 was found from fitting Eq. 1 to the data in Fig. 3C. A similar indication of cooperativity binding of H$^+$ was found for the flounder and rat isoforms (9).
The steady-state charge distribution (V₀.5 in Table 3) was shifted in the positive direction by low pH, which is opposite of what we observed when the Na⁺ concentration was lowered. This indicates that protons are not merely hindering Na⁺ from reaching the first binding site, since this would be expected to cause a shift of V₀.5 in the negative direction, as with lowering the Na⁺ concentration. One possible explanation is that protons interact with the first Na⁺ binding site, but in contrast to Na⁺, they are not able to support charge movement or steady-state transport. In this work, we limited the range of pH values examined to pH 7.4 and 6.6, since lowering the pH further caused marked instabilities in the holding currents for extreme potentials. Examining current records acquired at pH 5.0 using a narrower voltage range revealed that at this pH, pre-steady-state charge movements were completely suppressed and the transients looked like those obtained in water-injected control oocytes (not shown), as previously observed (9).

Earlier work on rat and flounder isoforms (9) examined the pH dependency of pre-steady-state charge movement over a significantly broader range. Over the pH range 8.0–5.6, V₀.5 was shifted in the positive direction in both the presence and absence of Na. However, in contrast to the human isoform, Q max was only minimally affected by reduced pH for both flounder and rat isoforms whether in the presence or absence of Na⁺. Furthermore, no pre-steady-state charge movement was observed at pH 5.0, presumably because at this low pH the steady-state charge distribution was shifted so far toward positive potentials that no charge movement could be detected in the voltage range normally possible with oocytes. From these studies, it was postulated that protons mainly interact with the transition of the empty carrier and with the binding of the last two Na⁺ ions. It appears that the human isoform differs from the rat and flounder isoforms in that the first Na⁺ binding step shows more proton sensitivity.

**Revised Kinetic Model for the NaPi-IIa Transport Cycle**

The results of the steady-state and pre-steady-state analysis in this study were used to modify the kinetic model originally proposed for NaPi-IIa (7, 9, 12). We simulated steady-state and pre-steady-state currents by assigning rate constants to the transitions between the states shown in Fig. 8A, as described earlier (7). Voltage-dependent rates were formulated according to Eyring transition rate theory, assuming sharp energy barriers. Similar to flounder and rat NaPi-II, voltage dependency was assigned to the transitions of the empty carrier (transitions 8–1), the first Na⁺ binding step (transitions 1–2), and the last Na⁺ debinding step (transitions 7–8). However, to simulate the voltage dependency of Kₘ(HPO₄²⁻), seen in Fig. 4, it was necessary to introduce voltage dependency for HPO₄²⁻ binding (transitions 2–3). In addition, the voltage-dependent rates associated with the Na⁺ binding steps (transitions 1–2 and 7–8) were simulated with asymmetrical energy barriers. Using the rate constants as defined (see legend for Fig. 8), we could simulate the voltage dependencies of the Pₐ-induced currents at variable Pₐ (100 mM Na) and variable Na⁺ (1 mM Pₐ) and reproduce the experimentally observed voltage dependency of Kₘ(HPO₄²⁻) and Kₙa. Thus a major difference from previous models (developed for the rat and flounder isoforms) is that HPO₄²⁻ binding is voltage dependent, indicating that HPO₄²⁻ ions bind in an area within the membrane electrical field.

In general, our model presented in Fig. 8 incorporates more skewness into rate constants and the factors that influence voltage-dependent transitions than previous models developed for the rat and flounder NaPi-II isoforms. Instead of assuming equal and slow forward and backward rate constants for the transition of the fully loaded carrier (transitions 4–5), the new model requires that, upon binding of all substrates on the external side, the fully loaded carrier (state 4) favors transitions 4–5 over the reverse reaction. Furthermore, we incorporated a larger voltage dependency for the last Na⁺ debinding step (transitions 7–8) than for the first Na⁺ binding step (transitions 1–2). Finally, whereas in previous models, mobile charges were assumed to move over symmetrically located barriers, here we found it necessary to introduce strong asymmetry into the first Na⁺ binding step (1–2) and the transition of the empty carrier (1–8) to obtain satisfactory agreement between the measured and simulated data.

The model presented in Fig. 8 is limited. We were not able to satisfactorily reproduce suppression of pre-steady-state charge movement (Q max) caused by reducing extracellular pH from 7.4 to 6.6. Since we obtained the data in Fig. 7 by integrating the full oocyte pre-steady-state current response, it is possible that a transient with a faster time constant could have been masked by the oocyte capacitative transient and thus account for the “missing” charge in the data at pH 6.6. Nevertheless, we introduced H⁺ modulation of the first Na⁺ binding, since the pH-dependent decrease in Q max is much larger in the presence of external Na⁺ than in its absence. Furthermore, from the slope V₀.5/log[Na] in Fig. 6D, we estimated that the first Na⁺ binding (transitions 1–2) would sense 60% of the transmembrane electrical field. In the model in Fig. 8, this value is set to α₁ = 0.2, and if we estimate this fraction from the slope of the simulated V₀.5/log[Na] data in Fig. 8F, the value is close to 1. The discrepancy most likely results from fitting one single Boltzmann function to a charge distribution containing contributions from three charge-carrying transitions (1–2, 7–8, and 8–1). Finally, little information is available about factors influencing the intracellular reaction steps (transitions 5–8), such as the order of substrate release and their affinities. These issues would be more easily resolved using methods, such as the cut-open oocyte technique, that allow a faster voltage clamp of the membrane as well as manipulation of intracellular ion concentrations.

**Conclusions**

We have examined the interactions of Na, P_i, and pH on the transport cycle of the human NaPi-IIa isoform. The interdependencies of the two substrates are complex, with the Na⁺ concentration affecting the Pi binding affinity and vice versa. The whole process is modulated by pH, which acts via titration of HPO₄²⁻, by reducing the Na⁺ affinity of one or more Na⁺ binding sites, and by modulating the transition of the empty carrier. The data have enabled us to modify previous models of NaPi-II transport by including proton modulation of the first Na⁺ binding step and voltage dependency of HPO₄²⁻ binding.

**GRANTS**

This work was supported by grants to H. Murer from the Swiss National Science Foundation, the Gebert Rüf Stiftung (www.gristiftung.ch), the Hartmann-Müller-Stiftung (Zürich), the Olga Mayenfish-Stiftung (Zürich), and the Union Bank of Switzerland.
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