The Voltage Dependence of a Cloned Mammalian Renal Type II Na\(^+\)/P\(_i\) Cotransporter (NaP\(_i\)-2)

IAN FORSTER, NATI HERNANDO, JÜRG BIBER, AND HEINI MURER

From the Physiologisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland

ABSTRACT The voltage dependence of the rat renal type II Na\(^+\)/P\(_i\) cotransporter (NaP\(_i\)-2) was investigated by expressing NaP\(_i\)-2 in Xenopus laevis oocytes and applying the two-electrode voltage clamp. In the steady state, superfusion with inorganic phosphate (P\(_i\)) induced inward currents (I\(_p\)) in the presence of 96 mM Na\(^+\) over the potential range \(-140 \leq V \leq +40\) mV. With P\(_i\) as the variable substrate, the apparent affinity constant (K\(_m\)) was strongly dependent on Na\(^+\), increasing sixfold for a twofold reduction in external Na\(^+\). K\(_m\) increased with depolarizing voltage and was more sensitive to voltage at reduced Na\(^+\). The Hill coefficient was close to unity and the predicted maximum I\(_p\) (I\(_\text{max}\)) was 40% smaller at 50 mM Na\(^+\). With Na\(^+\) as the variable substrate, K\(_m\) was weakly dependent on both P\(_i\) and voltage, the Hill coefficient was close to 3 and I\(_\text{max}\) was independent of P\(_i\) at \(-50\) mV. The competitive inhibitor phosphonoformic acid suppressed the steady state holding current in a Na\(^+\)-dependent manner, indicating the existence of uncoupled Na\(^+\) slippage. Voltage steps induced pre–steady state relaxations typical for Na\(^+\)-coupled cotransporters. NaP\(_i\)-2-dependent relaxations were quantitated by a single, voltage-dependent exponential. At 96 mM Na\(^+\), a Boltzmann function was fit to the steady state charge distribution (Q-V) to give a midpoint voltage (V\(_{0.5}\)) in the range \(-20\) to \(-50\) mV and an apparent valency of \(\sim 0.5\) e\(^-\). V\(_{0.5}\) became more negative as Na\(^+\) was reduced. P\(_i\) suppressed relaxations in a dose-dependent manner, but had little effect on their voltage dependence. Reducing external pH shifted V\(_{0.5}\) to depolarizing potentials and suppressed relaxations in the absence of Na\(^+\), suggesting that protons interact with the unloaded carrier. These findings were incorporated into an ordered kinetic model whereby Na\(^+\) is the first and last substrate to bind, and the observed voltage dependence arises from the unloaded carrier and first Na\(^+\) binding step.

KEY WORDS: ion cotransport • kinetics • steady state • pre–steady state relaxations • Xenopus laevis oocytes

INTRODUCTION

The reabsorption of inorganic phosphate (P\(_i\))\(^1\) at the epithelial brush border membrane lining the proximal tubule lumen of the mammalian kidney is the result of a secondary active transport process. This is mediated by a substrate-specific, cotransporter protein that couples a downhill Na\(^+\) flux to inward transport of P\(_i\) (reviewed in Murer et al., 1991, 1994; Murer and Biber, 1997). Two renal P\(_i\)-cotransporter types have been identified so far. The type II Na\(^+\)/P\(_i\) cotransporter is distinguished from the type I both at the molecular level and functionally by its higher P\(_i\) affinity, sensitivity to pH, regulation by external P\(_i\), and strong dependence on external Na\(^+\) (Murer and Biber, 1997). Moreover, under physiological conditions, type II Na\(^+\)/P\(_i\) transport kinetics are electrogenic, whereby each transport cycle involves a net transmembrane charge transfer (Busch et al., 1994). As a consequence of electrogenicity, if any step in the transport cycle carries charge across the membrane, then that step must be sensitive to the membrane potential, thereby giving rise to voltage-dependent kinetics.

Evidence for electrogenic Na\(^+\)/P\(_i\) cotransport was first reported by Hoffmann et al. (1976) and later confirmed by Béliveau and co-workers (Béliveau and Ibn-noul-Khatib, 1988; Béliveau and Strévey, 1991) using tracer flux techniques applied to isolated renal brush border membrane vesicles (BBMVs). Furthermore, Burkhart et al. (1981) demonstrated that P\(_i\) induced a change in membrane potential by preloading vesicles with a voltage-sensitive fluorescent dye. However, in all these studies the lack of direct control of the BBMV transmembrane potential has prevented precise characterization of the electrogenicity of Na\(^+\)/P\(_i\) cotransport.

Direct evidence for electrogenicity was obtained from microelectrode studies on intact proximal tubules, whereby addition of P\(_i\) to the luminal perfusate caused a depolarization of the epithelial membrane (Samarzija et al., 1980), consistent with a net inward

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\(^1\) Abbreviations used in this paper: BBMV, brush border membrane vesicles; I\(_{0p}\), oocyte holding current; I\(_{p}\), P\(_i\)-induced inward currents; I\(_{PFA}\), PFA-sensitive component; I\(_{V}\), current-voltage; NaP\(_i\)-2 rat type II Na\(^+\)/P\(_i\) cotransporter; PFA, phosphonoformic acid; P\(_i\), inorganic phosphate; V\(_{0.5}\), oocyte holding potential.

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flux of positive charge. More recently, Busch et al. (1994) characterized the electrogenicity by expressing the type II Na\(^+/P_i\) cotransporter (NaP\(_{2}\)), cloned from rat kidney in Xenopus laevis oocytes. They showed that in the mandatory presence of extracellular Na\(^+\), P\(_i\) induced an inward current (I\(_p\)) for membrane potentials (V) in the range −80 < V < +10 mV. Consistent with the findings from BBMVs, the magnitude of I\(_p\) depended on the substrate concentrations, the extracellular pH, and membrane potential. However, in contrast to the 2:1 stoichiometry for Na\(^+\)/P\(_i\) at pH 7.4 proposed from BBMV studies, a finding of a Hill slope close to 3 for the Na\(^+\) dose response at saturating P\(_i\) suggested a 3:1 stoichiometry for type II Na\(^+\)/P\(_i\) cotransport at −50 mV.

To develop comprehensive kinetic models of type II Na\(^+\)/P\(_i\) cotransport, account must be taken of the modulation of transport function by membrane potential, thereby necessitating identification of voltage-dependent partial reactions in the transport cycle. We now address this need by characterizing both the steady state and pre-steady state behavior of the NaP\(_{2}\) isoform over a wide membrane potential and substrate concentration range. We show that this mammalian isoform functions in a kinetically similar way to the flounder isoform (NaP\(_{2}\)-5) recently described by Forster et al. (1997a), but with significant differences in the detailed kinetics. Moreover, we have identified and characterized a Na\(^+\)-slippage component in type II Na\(^+\)/P\(_i\) cotransport.

**Materials and Methods**

**Oocytes**

Stage V-VI oocytes from the clawed frog *Xenopus laevis* were prepared according to standard procedures and injected with 10 ng/oocyte of cRNA encoding for the NaP\(_{2}\) protein (Werner et al., 1990) 24–48 h after defolliculation. Cells were incubated at 16–18°C in modified Barth’s solution (see below) and tested for expression 2–5 d after injection. Only cells having a resting membrane potential below −20 mV and a steady state leakage current <100 nA at −50 mV were used.

**Electrophysiology and Data Acquisition**

Oocytes were placed in a small recess in a plexiglas superfusion chamber (0.2 ml vol) and continuously superfused (5 ml/min) with ND96 control solution (see below). Computer controlled valves allowed fast and reproducible solution changes. All superfusates were cooled to 20–22°C before entering the chamber. Dose-response protocols were run with increasing concentration of the test substrate and the application time for P\(_i\) never exceeded 20 s to avoid possible loading of the cell. Long-term stability of the preparation was monitored using a chart recorder and each new test solution application was made only after the holding current had returned to the previous control value, with the test application always preceded by recording the response to the control solution. Oocytes were voltage clamped using a custom-built two-electrode voltage clamp with active series resistance compensation to improve the clamping speed. Furthermore, for the steady state recordings using the staircase protocol, an electronic transient subtraction stage was used to increase the ADC dynamic range to avoid overloading the data acquisition system. Cells were normally clamped at a holding potential of −50 mV to reduce possible contamination from Ca\(^{2+}\)-activated Cl\(^−\) currents at depolarized potentials. Current recordings were filtered using an eight-pole Bessel filter (902; Frequency Devices, Haverhill, MA) at a cut-off frequency less than twice the sampling frequency used. Data acquisition, voltage command generation, and solution valve control were done using laboratory built PC-compatible hardware and programmed using DATAC software (Bertrand and Bader, 1986).

**Solutions and Chemicals**

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Fluka (Buchs, Switzerland). Solutions were prepared as follows (mM/liter). (a) Oocyte incubation (modified Barth’s solution): 88 NaCl, 0.41 CaCl\(_2\), 0.82 MgSO\(_4\), 2.5 NaHCO\(_3\), 2 Ca(NO\(_3\))\(_2\), 7.5 Tris, pH 7.6, supplemented with antibiotics (10 mg/liter penicillin, streptomycin). (b) Control superfusate (ND96): 96 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES, titrated to pH 7.4 with NaOH. Isomolar BaCl\(_2\) was routinely substituted for CaCl\(_2\) to reduce contamination from endogenous Ca\(^{2+}\)-activated Cl\(^−\) currents that were observed for V > −10 mV and allow a greater range of P\(_i\) concentrations, except for experiments involving phosphonoformic acid (PFA), which otherwise complexes with Ba\(^{2+}\). For Na\(^+\)-substitution experiments, N-methyl-D-glucamine replaced Na\(^+\) at the appropriate concentration to maintain isosmolar external solutions. Solutions were titrated with HCl or KOH to pH 7.4. (c) Test superfusate: inorganic phosphate such as Na\(_2\)HPO\(_4\), 6H\(_2\)O, was added to the solutions in b, and pH was adjusted to 7.4. For the Na\(^+\)-substitution experiments, a KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (pH 7.4) was used to minimize changes in Na\(^+\) concentration. (d) PFA experiments: to take account of PFA being a trisodium salt, Na\(^+\) was added in the appropriate concentration to the control solutions to give equal final Na\(^+\) concentrations.

**Data Analysis and Curve Fitting**

Preliminary data analysis was performed using macro routines written in the DATAC language (Bertrand and Bader, 1986). Nonlinear regression analysis was performed using Inplot v. 4.0 or Prism v. 2.0 software (Graphpad Inc., San Diego, CA). All data are shown as mean ± SEM (n), where n is the number of oocytes for a particular protocol. Experimental protocols were repeated at least twice on different batches of oocytes from different frogs. Exponential curve fitting was performed using a Chebychev transform routine written in C.

**Dose-response curves**

Responses with respect to a variable substrate S, were quantified as peak P\(_i\)-induced current and a form of the Hill equation was fit to the dose response:

\[
I_p = I_{p,max} [S]^n / [1 + [S]^n + (K_s)^n],
\]

where [S] is the substrate concentration, \(I_{p,max}\) the extrapolated maximum current, \(K_s\) the concentration of substrate S that gives a half maximum response or apparent affinity constant, and \(n\) the Hill coefficient.

**Two-state Eyring-Boltzmann model for transmembrane charge movements**

For a two-state system in which \(N\) charged entities, each having an apparent valency \(z\), can translocate independently between two states within the transmembrane field, the macroscopic steady state charge distribution as a function of transmembrane voltage \(V\), is given by:

\[
Q = Q_{0,up} + Q_{0,down} / [1 + \exp \left( -ze(V - V_{1/2}) / (kT) \right) ] ,
\]

where \(Q_{0,up}\) and \(Q_{0,down}\) are the steady state charge fluxes for the upper and lower state, respectively, \(ze\) is the elementary charge times the valency of the carrier, \(V_{1/2}\) is the half maximum voltage, and \(k\) is the Boltzmann constant.
where \( Q_{\text{max}} = Nze \), the maximum charge translocated, \( Q_{\text{app}} \) which depends on the holding potential, is the charge translocated at the hyperpolarizing limit, \( V_{0.5} \) is the voltage at which the charge is distributed equally between the two states, \( e \) the electronic charge, \( k \) Boltzmann’s constant, and \( T \) the absolute temperature.

**Simulations.** To simulate pre–steady state and steady state currents, the differential equations describing the state transitions were solved for the state occupancies by using matrix methods to find the eigenvalues and eigenvectors (e.g., Press et al., 1992). For any transition between states \( i \) and \( j \) involving an apparent charge movement \( \delta \), the forward and backward rate constants were expressed as \( k_f = K_{f,i,j} \exp(-\delta eV/kT) \) and \( k_b = K_{b,j,i} \exp(\delta eV/kT) \), respectively, where \( K_{f,i,j} \) and \( K_{b,j,i} \) are the corresponding forward and backward rate constants, respectively, at \( V = 0 \) and \( \delta \) is an asymmetry factor (0 ≤ \( \delta \) ≤ 1) that defines the relative position of the energy barrier within the transmembrane electric field (e.g., Adrian, 1978). For transitions involving substrate binding, the rate constant was scaled by the factor \( S \), where \( S \) is the substrate concentration and \( n \) the number of ions involved in the binding reaction. Simulation routines were written in C and adapted from those given in Press et al. (1992). For simulations, the temperature was assumed to be 20°C.

**RESULTS**

**Voltage Dependence of \( P_i \) Dose Response in the Steady State**

Fig. 1A shows the typical phosphate-induced current at two holding potentials (\( V_h \)) recorded from an oocyte expressing NaPi-2 when the control superfusate was rapidly switched to a test solution containing 1.0 mM \( P_i \). The dependence on \( V_h \) of the maximum steady state response confirmed that NaPi-2 exhibited electrogenic behavior. Such currents were not observed when the same protocol was applied to water or noninjected oocytes (data not shown), as reported previously (Busch et al., 1994). With fast superfusion of the oocyte, the \( P_i \)-induced response gave a rapid initial phase, the rise time of which was limited by the recording bandwidth. This was followed by a slower relaxation phase before finally reaching a steady state level after ~10–15 s. Washout of \( P_i \) was also accompanied by a similar biphasic return to the baseline. At all potentials tested, the magnitude of the fast phase was proportional to \( V_h \). We attributed the slower phase to the electrogenic response of regions of the oocyte membrane that did not experience an initial rapid exchange of superfusate due to unstirred layer effects. These oocyte-dependent response kinetics were not investigated further in this study. Once the maximum was reached, \( I_p \) usually remained constant for intervals exceeding 20 s.

To obtain the steady state current–voltage (I–V) relationship, it was convenient to apply a voltage staircase in the range −140 to +40 mV after the current at \( V_h \) had stabilized. The duration of each step (50 ms) was chosen so that a steady state was reached before the next voltage transition. This was also confirmed by checking that \( I_p \) at different holding potentials was the same as the step current corresponding to the same potential (data not shown). Some preliminary experiments were also performed using a continuous ramp of 1-s duration and the results were indistinguishable from the staircase (Forster et al., 1996).

![Figure 1](image-url)
Fig. 1B shows the typical response to a voltage staircase, starting after \( I_p \) had stabilized, in the absence and presence of 1.0 mM \( P_i \) in the superfusate. The small transient at each transition indicates incomplete suppression of the endogenous oocyte capacitive transient (see Materials and Methods) due to \( P_i \) suppression of the pre–steady state response. The I-V relationship for the \( P_i \)-dependent current was obtained directly from the difference between these records, as shown in the bottom trace. In the physiological range of potentials \((-60 < V < -20 \text{ mV})\), the I-V relation was linear, whereas at strong depolarizing and hyperpolarizing potentials, it deviated from linearity. This behavior suggested the presence of rate-limiting, voltage-independent steps in the transport mechanism.

To characterize further the \( \text{NaP}_{2} \) voltage dependency in the steady state, we applied the staircase protocol with different \( P_i \) and fit Eq. 1 to the dose-response data at each potential. The resulting family of I-V data for a typical cell at 96 mM Na\(^{+}\) is shown in Fig. 2A for six \( P_i \) values in the range 0.006–1 mM. Typically, no current reversal was observed, although with some oocytes at low \( P_i \) (<0.01 mM) the \( P_i \)-induced current did show reversal at potentials <0 mV. This behavior was not reproducible, but appeared to be dependent on the oocyte batch/donor frog. These apparent outward cur-

![Figure 2](image-url)
rents at $-140 \text{ mV}$ typically did not exceed 5% of the peak induced current at 1 mM $P_i$ and most likely reflect the instability of the preparation as they would become more apparent when subtracting two quantities of similar magnitude. We derived the steady state $P_i$ dose response with respect to the magnitude of $I_p$ from these data at the same $V_h$ ($-50 \text{ mV}$) for two $Na^+$ concentrations (96 and 50 mM) and the same oocyte (Fig. 2 B). The form of the dose-response relationships resembled a rectangular hyperbola with clear evidence of saturation at high $P_i$. Fitting Eq. 1 to these data indicated that, for a reduction in $Na^+$ from 96 to 50 mM, the predicted maximum induced current ($I_p^{\text{max}}$) was significantly reduced by $38 \pm 5\%$ ($N = 5$) at 50 mM $Na^+$, when comparing the responses from the same five oocytes. Moreover, the fit indicated that the half-maximum concentration for $P_i$ ($K_{P_i}$) increased from $0.057 \pm 0.006$ mM at 96 mM $Na^+$ to $0.35 \pm 0.03$ mM at 50 mM $Na^+$ for the same five oocytes. The shift in $K_{P_i}$ is seen more clearly by normalizing the data to $I_p^{\text{max}}$ and plotting on a semilogarithmic scale (see Fig. 2 B, inset). Finally, in agreement with previous results (Busch et al., 1995; Hartmann et al., 1995), the estimated Hill coefficient ($n$) was close to unity for both concentrations (at 96 mM $Na^+$, $n = 0.96 \pm 0.05$ and at 50 mM $Na^+$, $n = 0.92 \pm 0.06$).

From the Hill equation fits, we determined the potential dependence of the estimates for $n$, $K_{P_i}$, and $I_p^{\text{max}}$, pooled from representative cells from different donor frogs (Fig. 2 C). Reliable estimates of current were restricted to voltages $<0$ mV for both $Na^+$ concentrations. The Hill coefficient was close to unity for each $Na^+$ concentration and showed little dependence on $V$ (over the range $-140 \leq V \leq 0$ mV, at 96 mM $Na^+$, $n = 0.96 \pm 0.05$ and at 50 mM $Na^+$, $n = 0.92 \pm 0.06$).

**Figure 3.** Steady state voltage dependency of $Na^+$/Pi transport as a function of $Na^+$ concentration. (A) Typical family of I-V curves obtained from one cell with 1 mM $P_i$ and six $Na^+$ concentrations as indicated (millimolar). Data points are joined for visualization only. (B) Typical dose-response data for the same cell at two $P_i$ concentrations: 1.0 ($\square$) and 0.1 ($\square$) mM with $Na^+$ as the variable substrate and $V_h = -50$ mV. Eq. 1 was fit to the data points. For this cell, the fit parameters were: (1 mM $P_i$) $K_{Na}^{\text{app}} = 50.1$ mM, $n = 2.6$, $I_p^{\text{max}} = 109$ pA; and (0.1 mM $P_i$) $K_{Na}^{\text{app}} = 89$ mM, $n = 2.6$, $I_p^{\text{max}} = 114$ pA. (C) Summary of voltage dependence of fitted parameters comparing data for two concentrations of $P_i$: 1 ($\square$) and 0.1 (■) mM. Data are shown as mean ± SEM. Only SEMs exceeding symbol size are shown. Data points are joined for visualization only. (C, I) $n =$ Hill coefficient, (dashed line) $n = 3$; (C, 2) $K_{Na}^{\text{app}} =$ apparent affinity constant; (C, 3) $I_p^{\text{max}}/I_p^{\text{max}(-100)} =$ maximum induced current normalized to the value at $-100$ mV.
1.0 ± 0.01 and at 50 mM Na+, n = 0.87 ± 0.03) (Fig. 2 C, top). At 50 mM Na+, n was significantly smaller than at 96 mM Na+, particularly at higher V. At 96 mM Na+, $K_{m}^{Na}$ (Fig. 2 C, middle) was weakly voltage dependent and increased by ~60% over the voltage range from $V = -140$ to $V = +20$ mV. At 50 mM Na+, the increase in $K_{m}^{Na}$ was fourfold over the same range of $V$, with a significant change occurring in the physiological range ($-70 < V < -20$ mV). Finally, the voltage dependence of $I_{\text{pmax}}$ for the two Na$^{+}$ concentrations superimposed when normalized to the current at −100 mV (Fig. 2 C, bottom), indicating that the voltage dependence of $I_{\text{pmax}}$ was the same for the two Na$^{+}$ concentrations tested at saturating $P_{i}$.

**Voltage Dependence of Na$^{+}$ Dose Response in the Steady State**

Next we studied the voltage dependency of NaPi-2 transport as a function of external Na$^{+}$ with fixed $P_{i}$, using the same methods as above. Fig. 3 A shows a typical set of I-V curves for the same oocyte with 1 mM $P_{i}$ and six Na$^{+}$ concentrations. For Na$^{+}$ > 25 mM, the I-V curves showed no current reversal up to +40 mV. For this particular cell at 10 mM Na$^{+}$, the $P_{i}$-induced current reversed at −40 mV. As noted above, the variability of this apparent reversal reflects the precision of the subtraction/recording procedure and should not be taken as a true indication of current reversal. We generated I-V curves in response to the staircase protocol at two $P_{i}$ concentrations: 1 mM (close to saturating $P_{i}$) and 0.1 mM (close to $K_{m}^{Na}$). We were unable to make reliable determinations of the I-V relations at $P_{i} < 0.1$ mM and Na$^{+} < 50$ mM because of the small magnitude of the induced currents (typically < 10 nA), the estimation of which was sensitive to any drift in the endogenous holding current during the course of the experiment.

Fig. 3 B shows the typical steady state dose response for the same cell with the two $P_{i}$ concentrations at −50 mV holding potential. Plotted on a linear abscissa, the dose-response relationship was sigmoidal with an inflection at low Na$^{+}$, typical for cooperative substrate binding and consistent with our previous results (Busch et al., 1994, 1995). Fitting Eq. 1 to these data was less reliable than for the $P_{i}$ dose dependency determination because of the absence of clear saturation at the maximum Na$^{+}$ concentration possible, particularly at 0.1 mM $P_{i}$. We attempted to superpose the oocytes with Na$^{+}$ > 120 mM for short periods, but the hyperosmotic conditions resulted in significant holding current instability. Despite this limitation, the results of fitting Eq. 1 to these data suggested that the predicted $I_{\text{pmax}}$ was independent of $P_{i}$ (at $V_{h} = -50$ mV, the ratio $I_{\text{pmax}}[1$ mM $P_{i}] / I_{\text{pmax}}[0.1$ mM $P_{i}] = 1.08 ± 0.03, N = 5$). Furthermore, at $V_{h} = -50$ mV, the estimated Hill coefficient was close to 3 in both cases (at 0.1 mM $P_{i}$, n = 2.9 ± 0.1 and at 1 mM $P_{i}$, n = 2.9 ± 0.2, N = 5), whereas the apparent affinity constant for Na$^{+}$ ($K_{m}^{Na}$) was clearly $P_{i}$ dependent. For the same five cells at $V_{h} = -50$ mV with 1 mM $P_{i}$, $K_{m}^{Na}$ was 52.0 ± 2.2 mM and with 0.1 mM $P_{i}$, $K_{m}^{Na} = 81.8 ± 2.3$ mM.

Fig. 3 C shows the voltage dependence of the three fit parameters ($K_{m}^{Na}$, n, and $I_{\text{pmax}}$) for the two $P_{i}$ concentrations, pooled from oocytes from different donor frogs. For both $P_{i}$, n (Fig. 3 C, top) remained approximately voltage independent over the physiological range of potentials (at 1.0 mM $P_{i}$, n = 2.81 ± 0.04 and at 0.1 mM $P_{i}$, n = 2.89 ± 0.08). Furthermore, at 0.1 mM $P_{i}$, $K_{m}^{Na}$ (Fig. 3 C, middle) increased monotonically with depolarizing membrane potential, whereas at 1 mM $P_{i}$, the voltage dependence was less marked and the normalized voltage dependence of $I_{\text{pmax}}$ for the 10-fold reduction in $P_{i}$ showed a small but consistent deviation as $V$ approached 0 mV (Fig. 3 C, bottom). This suggested that the voltage sensitivity of the rate limiting step(s) was reduced at the lower $P_{i}$ for saturating Na$^{+}$.

**Suppression of $I_{h}$ by Phosphononoformic Acid Reveals a Na$^{+}$-dependent Current in the Absence of External $P_{i}$**

We tested for the presence of slippage in the type II Na$^{+}$/P$_{i}$ system using PFA, a competitive inhibitor of Na$^{+}$/P$_{i}$ cotransport (Busch et al., 1995; Kempson, 1988). Fig. 4 A shows measurements of the holding current ($I_{h}$) at $V_{h} = -50$ mV made at 5-s intervals for a typical oocyte expressing NaPi-2 and a noninjected oocyte from the same batch, in response to the application of substrate combinations indicated. For NaPi-2, in 105 mM Na$^{+}$, 0.3 mM P$_{i}$ induced an increase in $I_{h}$ of 89 nA, whereas in the presence of 3 mM PFA, $I_{h}$ increased by only ~9 nA, which confirmed the inhibitory effect of PFA. In contrast, the noninjected oocyte showed a 2-nA change in the presence of 0.3 mM P$_{i}$, which might be attributable to an endogenous Na$^{+}$/P$_{i}$ cotransporter, and a similar change also occurred in the presence of PFA. For both cells, in the absence of P$_{i}$, switching Na$^{+}$ between 9 and 105 mM led to a concomitant change in $I_{h}$. This would be expected if a component of $I_{h}$ were due to a Na$^{+}$ conductance, although in the case of NaPi-2, the change was fivefold larger. Moreover, for cells expressing NaPi-2, 3 mM PFA suppressed $I_{h}$ at 105 mM Na$^{+}$ by ~50% and induced the same relative decrease at 9 mM Na$^{+}$, whereas no measurable shift in $I_{h}$ occurred for the noninjected cell. This behavior would be consistent with the PFA-sensitive component ($I_{\text{PFA}}$) being due to a Na$^{+}$ conductance. If this were the case, we would predict a shift in the reversal potential ($E_{r}$) for $I_{\text{PFA}}$ in response to a change in external Na$^{+}$. Fig. 4 B shows a typical I-V relation for $I_{\text{PFA}}$ for two external Na$^{+}$ (109 and 59 mM), obtained by subtracting the response to a staircase voltage protocol in the presence of 3 mM PFA from the response in the absence of PFA.
The expected shift towards a more negative $E_r$ was found and, moreover, this behaved in a Nernstian manner for the three $\text{Na}^+$ concentrations tested, giving a slope of $64.4 \pm 1.7 \text{ mV}$. We were unable to determine $E_r$ reliably for $\text{Na}^+ < 50 \text{ mM}$ due to the small magnitude $I_{\text{PFA}}$. The $\text{Na}^+$ dependence of this component was characterized further by determining the dose dependency at $-50 \text{ mV}$, as shown in Fig. 4C for a typical cell expressing NaPi-2. The relation was nonlinear, indicating saturation of this pathway and, when Eq. 1 was fit to these data, we obtained a $K_m = 128 \text{ mM}$ and a Hill coefficient of 0.92 ($n = 1$). Finally, the magnitude of $I_{\text{PFA}}$ correlated linearly with the $P_i$-induced current for a number of oocytes from different batches and with different levels of expression, indicating that $I_{\text{PFA}}$ was $\sim 12\%$ of the $P_i$-induced current at 1 mM $P_i$ (Fig. 4D).

**Figure 4.** Characterization of $P_i$-independent current component using phosphonoformic acid. (A) Oocyte holding current ($I_h$) at $V_h = -50 \text{ mV}$ for continuous superfusion with the indicated solutions for a NaPi-2-expressing oocyte (top) and noninjected oocyte (bottom) from the same batch of oocytes and recorded during the same experimental session. $I_h$ was sampled at 5-s intervals and the sample points have been joined by straight lines for clarity. Each superfusate combination was applied for 1 min to allow a stable baseline to be reached. Top and bottom dashed lines, superimposed on NaPi-2 data, indicate $I_h$ at 9 and 105 mM $\text{Na}^+$, respectively. (B) Typical I-V relations for the PFA-sensitive component at two concentrations of $\text{Na}^+$: 109 (■) and 59 (▲) mM. A staircase voltage protocol with 5-mV, 100-ms-long steps was applied to the oocyte. Points represent the difference between the steady state current at the end of each step under control conditions and the response in the presence of 3 mM PFA. As PFA is a trisodium salt, the control solution $\text{Na}^+$ concentration in each case was adjusted to ensure that the $\text{Na}^+$ gradient remained the same. Continuous lines are polynomial fits to the data, used to determine the reversal potential ($E_r$). Inset shows $E_r$ plotted as a function of $\text{Na}^+$ concentration. Note that $\text{Na}^+$ concentration is plotted on a log$_{10}$ scale. Number of cells is indicated for each $\text{Na}^+$ tested. Straight line is a linear regression giving a slope $64.4 \pm 1.7 \text{ mV}$. (C) Dose dependency with respect to $\text{Na}^+$ for $I_h$ in the absence of $P_i$ at $V_h = -50 \text{ mV}$. Data shown for a typical cell expressing NaPi-2. Each point represents the induced change in steady state current, relative to 0 mM $\text{Na}^+$. Continuous line is the fit using Eq. 1, giving $K_m = 128 \text{ mM}$, $I_{\text{max}} = 74 \text{ nA}$, and $n = 0.92$. (D) The PFA-sensitive current correlates with the $P_i$-induced current. Data shown for 22 cells from several donor frogs displaying different levels of expression of NaPi-2. For each cell, the $P_i$-induced current ($I_p$) at 1 mM $P_i$ was determined together with the PFA-sensitive component ($I_{\text{PFA}}$) for 3 mM PFA at $V_h = -50 \text{ mV}$. The straight line is a linear regression line forced through the origin with a slope: $0.126 \pm 0.004$. 
Voltage Steps Induce Pre–Steady State Relaxations Typical of Na⁺-coupled Cotransporters

Voltage steps induced pre–steady state relaxations in oocytes expressing NaPi-2 (Fig. 5 A). The speed of relaxation to the steady state after a voltage step depended on the presence of Pₐ in the superfusate. In the absence of Pₐ (Fig. 5 A, top), pre–steady state relaxations, superimposed upon the normal capacitive charging transient, were observed when the membrane potential of NaPi-2-expressing oocytes was stepped from a holding potential, Vₕ = −100 mV. Relaxations were significantly suppressed when the cell was superfused with saturating Pₐ (3 mM) in the presence of 96 mM Na⁺ (Fig. 5 A, middle) and were absent in noninjected oocytes from the same batch (bottom). Apart from the difference in the steady state current at the test potential, the

as in A for holding potentials (Vₕ) −100, −40, and 0 mV for the same cell. Filled symbols are mean ± SEM of the ON transition τₛ at the three Vₕ. The straight lines represent the mean of the OFF relaxation τₛ over the whole voltage range: (dotted line) Vₕ = −100 mV, (dashed line) Vₕ = 0 mV. The mean OFF τₛ are: 7.5 ± 0.06 ms (Vₕ = −100 mV); 7.6 ± 0.05 ms (Vₕ = −40 mV), and 7.13 ± 0.14 ms (Vₕ = 0 mV). (B) Voltage dependency of charge movement (Q) associated with NaPi-2-related component at different Vₕ for the same cell as in B. (■) ON, (□) OFF. Continuous lines are fits of the Boltzmann function (Eq. 2) to the data. See Table I for fit parameters. (E) Correlation between estimated charge available for translocation at −100 mV (Q₋₁₀₀) and Pₐ-induced current at −100 mV (Iₚ₋₁₀₀) with 96 mM Na⁺ and 1 mM Pₐ. Data points are from 11 cells from different oocyte batches. Q₋₁₀₀ was obtained by fitting the Boltzmann function (Eq. 2) to the Q-V data and Iₚ₋₁₀₀ was obtained from steady state response of same cell under same recording conditions. Straight line is a linear regression line with slope 46 s⁻¹ and forced to intercept the origin.
records for the noninjected cell and NaPi-2 with 3 mM Pi appeared very similar.

To establish further that these relaxations were specifically related to the expression of NaPi-2, we examined the effect of superfusing with the inhibitor PFA. Fig. 5 B compares the current induced by a voltage step from −100 to 0 mV for superfusion with 3 mM PFA and 1.0 mM Pi. PFA suppressed the pre–steady state relaxation when compared with the control condition, analogous to the suppression by 1.0 mM Pi alone.

Exponential curve fitting allowed separation of the intrinsic oocyte charging component from the relaxation related specifically to NaPi-2. The fits were quantitated in terms of the time constant (τ) and charge transfer (Q) estimated from the product of τ and the amplitude of the NaPi-2-specific component extrapolated to time of respective voltage step onset. Biexponential curve fitting consistently showed that the faster (intrinsic) τ varied little with voltage (typically 0.6 ± 0.1 ms for −140 < V < +60 mV) or with the direction of the voltage step (data not shown). Often, however, when tuning the voltage clamp for fastest response, we observed that the intrinsic capacitive transient displayed an additional tail component with a τ ≈ 1 ms (see Fig. 7 B, inset), which was also observed in control oocytes from the same batch. Superfusion of control oocytes with 20 μM ouabain did not alter this component, suggesting that it did not arise from intrinsic Na/K pump (Holmgren and Rakowski, 1994). To ensure that any such intrinsic components did not influence the fit accuracy, the Pi-suppressed relaxation was characterized by fitting a single exponential, starting ~5 ms after the step. Fig. 5 C shows the voltage dependence of the slower τ obtained from a typical oocyte for three holding potentials (Vh). The ON transition relaxation kinetics showed a bell-shaped relation expected for voltage-dependent charge movements, whereas those for the corresponding OFF transition were independent of the voltage reached before returning to the holding potential. Moreover, the OFF transition τ at each Vh coincided with the interpolated value for the ON transition at that test voltage.

Fig. 5 D shows the voltage dependence of the corresponding steady state charge transfer for the same oocyte. These data indicate that the charge transfer tended to saturate at strong depolarizations. This suggested that the detected relaxation behavied as expected for movement of a fixed number of translocatable charges within the transmembrane field. Furthermore, we consistently observed that charge balance for the corresponding ON and OFF transitions occurred only over the mid-range of test potentials: the magnitude of the QON and QOFF deviated at extreme test potentials that could not be simply attributed to random error in the fit. The continuous lines in Fig. 5 D are fits using the Boltzmann equation (Eq. 2) to give parameters that facilitated comparison of the pre–steady state currents under different conditions. Although the lack of clear saturation at extreme potentials made fitting error prone, the fits at different Vh revealed several consistencies (Table I): the apparent valency was independent of Vh, and the mid-point voltage (V0.5), equivalent to the potential at which half the available charge was translocated, varied little with Vh, as did the total charge transfer, Qmax.

Finally, at Vh = −100 mV, the charge available for translocation from −100 mV, predicted from the Q-V fit (equivalent to the Q-V asymptote for V >> 0 and Vh = −100 mV), correlated linearly with I0 at −100 mV for several oocytes having different apparent NaPi-2 expression levels (Fig. 5 E). The slope was 46 s⁻¹, and this parameter was used to estimate the transporter turnover (see Discussion).

Substrate Dependence of Pre–Steady State Relaxations

As both substrates carry charge and could influence directly or indirectly the observed charge movements, we characterized the substrate dependence of relaxation kinetics and steady state charge distribution to identify the origin of pre–steady state relaxations.

Dependence on Na⁺ in the absence of Pi. Fig. 6 A shows representative records of pre–steady state relaxations for a cell expressing NaPi-2 with external Na⁺ varying from 96 to 0 mM in response to a voltage jump from −100 to 0 mV. For comparison purposes, the pre–steady state response for the same cell when superfused with 3 mM Pi and 96 Na⁺ is also shown. These data indicate: (a) for both the ON and OFF transitions, a component of the total pre–steady state charge movement was contributed by the presence of Na⁺ that appeared to diminish with decreasing Na⁺; (b) in the absence of external Na⁺, a NaPi-2-related charge movement was still present; and (c) under saturating Pi, the residual charge was further suppressed. This residual component was most likely not an oocyte-intrinsic charge movement since it was not observed in recordings from noninjected cells from the same oocyte batch (data not shown).

| Table I |
|---|
| The Effect of Holding Potential on the Steady State Charge Distribution |
| Vh (mV) | −100 | −40 | 0 |
| Transition | ON | OFF | ON | OFF | ON | OFF |
| z | 0.45 | 0.50 | 0.43 | 0.53 | 0.45 | 0.45 |
| V0.5 (mV) | −22 | −50.3 | −28.7 | −58.4 | −34.3 | −62.1 |
| Qmax (nC) | 5.70 | 4.25 | 5.00 | 4.53 | 4.63 | 5.62 |

Parameters obtained from a fit of the Boltzmann function (Eq. 2) to the Q-V data for the ON and OFF transitions (n = 1).
The voltage dependence of the NaPi-2-related charge movement and relaxation kinetics are summarized in Fig. 6, B and C, respectively. The Q-V data from a representative cell (Fig. 6 B) indicate that for a given voltage step from $V_h = -100$ mV a decrease in translocated charge occurred with reduced Na$^+$. If these data are normalized to the predicted Q$_{\text{max}}$ at 96 mM Na$^+$ and offset to superimpose at the depolarizing limit, $V_{0.5}$ shifted negatively with decreasing Na$^+$ (see Fig. 6 B, inset). Moreover, the $\tau$-V data, pooled from four representative cells (Fig. 6 C) showed the same general form of voltage dependence, but with an indication that the maximum $\tau$ was slightly reduced at the lower Na$^+$. Curve fitting was less reliable at low Na$^+$ because of the poorer signal-to-noise ratio and as a result we did not attempt fitting at 10 or 0 mM Na$^+$. The pooled results of the Boltzmann fit to the ON charge movement are summarized in Table II: the apparent valency, $z$, varied little with Na$^+$, whereas there was relative mean shift of $-25$ mV in $V_{0.5}$ with a fourfold reduction in Na$^+$, and the predicted Q$_{\text{max}}$ decreased by only $\sim 20\%$ over the same range.

**Dependence on P$_i$ with 96 mM Na$^+$.** Pre–steady state relaxations induced by a voltage step from $V_h = -100$ to 0 mV, observed in the absence of P$_i$, were progressively suppressed with increasing P$_i$ (Fig. 7 A). The voltage dependence of the NaPi-2 relaxation was not affected by increasing P$_i$ up to 0.1 mM with no consistent shift in $V_{0.5}$ and no change in the $\tau$-V (data not shown). For P$_i \geq 0.1$ mM, the relaxations were too small to fit reliably over the entire voltage range. To characterize the effect of P$_i$ further and facilitate quantification of the
relaxations, we subtracted the corresponding relaxation under saturating conditions (3 mM Pi) from the record at the test Pi for test potentials in the range −100 to +80 mV (Fig. 7 B). The validity of this procedure was based on the assumption that a saturating concentration of Pi would fully suppress the relaxations (see Fig. 5 A). The resulting difference record should then have the oocyte endogenous charging transient eliminated and provide a measure of the amount of charge suppressed by the respective Pi superimposed on the steady state Pi-induced current. When we attempted to fit a single exponential to the Pi-dependent relaxations, this gave a significantly worse fit compared with a bi-exponential fit, although at large Pi, where most of the apparent translocatable charge was suppressed, the concomitantly poorer signal-to-noise ratio made such fitting more ambiguous. The fitting revealed a fast component (typical τ = 700–1,000 μs) with a weak voltage dependence and a slower component that corresponded to the main relaxation observed in the unmanipulated records. We were unable to detect any significant dependence on Pi for either component. The rapid speed of capacitive charging for this cell (see Fig. 6 B, inset) with a main t = 191 ms, indicated that after 1 ms most of the membrane charging was complete and that the faster relaxation, revealed by the subtraction procedure, was most likely a true component of the total pre–steady state relaxation. To quantify the associated charge transfer, we integrated the total relaxation, commencing 1 ms after the voltage step. We also noted that the charge balance over the whole voltage range was consistently improved with the subtraction technique, resulting in <10% error in charge balance for −140 ≤ V ≤ +60 mV. The charge suppressed by Pi for four target potentials is shown in Fig. 7 C, and a competition curve (Eq. 1, with n = −1) was fit to the data points to give an apparent KdPi as a

**Figure 7.** The effect of changing external Pi on pre–steady state relaxations. (A) Pre–steady state relaxations recorded from a cell expressing NaPi-2 for a voltage jump from −100 to 0 mV for 0, 0.01, 0.03, 0.06, 0.1, 0.3, 1, and 3.0 mM Pi. The initial baseline shift reflects the steady state holding current induced by each Pi. Pi was applied in increasing concentration. Between successive applications, the cell was allowed to recover in 0 mM Pi until the initial steady state holding current at −50 mV was reestablished. (B) The Pi-suppressed current obtained by subtracting the record at 3 mM Pi from the test record under the same voltage step conditions as A and same cell. Each trace has been baseline-adjusted to the steady state value. The first millisecond of each difference record during the voltage transition was blanked. This corresponds to the time to complete most of the oocyte capacitive charging as indicated in the inset for a voltage jump from −50 to −40 mV, plotted on the same time scale (same cell). (C) Pi dose response for the same cell showing amount of apparent charge suppressed (Qsupp) as a function of Pi for five target potentials as indicated. Charge was estimated by numerical integration of the records as in B, with the steady state holding current suppressed. A small error is expected in Qsupp due to starting the integration at 1 ms. Continuous lines show fits with Eq. 1 for n = −1. (D) Voltage dependence of apparent Kd for suppression of pre–steady state charge found from fitting Eq. 1 to the data, for the same cell as in C.
function of the target potential (Fig. 7D). This indicated that the Pi concentration required to suppress 50% of the available charge at \( V_h = -100 \text{ mV} \) was relatively voltage independent.

Dependence on pH. H+ ions might also contribute to the pre–steady state relaxations since, for some Na+-coupled cotransporters, they are also known to act as a substrate (e.g., Hirayama et al., 1994). To test this hypothesis, we first investigated the effect of varying pH on pre–steady state relaxations with 96 mM Na+. As shown in Fig. 8A for voltage steps to three test potentials, a reduction in external pH from 7.4 to 6.2 caused a clear suppression of the relaxations both for the ON and OFF transitions, and the corresponding reduction in \( I_p \) was 81%. Quantification of the relaxations by single exponential curve fitting to the main relaxation revealed a consistent change in the relaxation voltage dependence as pH was reduced from 7.4 to 6.2: \( V_{0.5} \) of the Q-V curve shifted towards depolarizing potentials (Fig. 8B) and the relaxations slowed significantly for \( V > 0 \) in the \( \tau \)-V data (Fig. 8C). As shown in Fig. 8D, when Na+ was removed from the external medium changing pH from 7.4 to 6.2 further suppressed the relaxations; however, their small magnitude under these conditions prevented further quantification. No Pi-induced transport was detected in the steady state in the absence of external Na+ at these pH values (data not shown).

**DISCUSSION**

**Steady State Behavior**

Comparison with previous results. Our characterization of the steady state kinetics of the rat Na+/Pi cotransporter isoform, NaPi-2, gave findings consistent with those reported in previous electrophysiological studies (Busch et al., 1994, 1995; Hartmann et al., 1995). In these studies, kinetic parameters were determined over a limited potential range and no definitive conclusions regarding the voltage dependence of kinetics could be drawn. At \( V_h = -50 \text{ mV} \), we consistently measured a sixfold lower \( K_m^{Pi} \) compared with the value (0.31 mM) previously reported by Busch et al. (1994). The reason for this is unclear, although subsequent papers by this group characterizing other mammalian isoforms have also reported lower \( K_m^{Pi} \) (Busch et al., 1995; Hartmann et al., 1995), in agreement with our present findings. For both isotope flux and electrophysiological measurements, a Hill coefficient of unity at neutral pH for the Pi dose response is a consistent finding, providing strong evidence for a 1:1 stoichiometry for Pi. In the present case, we observed a Hill coefficient <1 at 50 mM Na+, which may reflect a systematic error in estimating \( I_p \) at levels close to or smaller than the endogenous oocyte currents.

Despite the lack of saturation of the Na+ dose response, our estimate for \( K_m^{Na} \) lies within the range also reported for BBMV flux assays (Hoffmann et al., 1976), where larger Na+ concentrations could be used, and previous electrophysiological studies on oocytes (Busch et al., 1994, 1995). However, in contrast to previous findings on BBMVs, we found that \( K_m^{Na} \) was dependent on Pi (Gmaj and Murer, 1986; see also Béliveau and Strévey, 1988). Furthermore, from BBMV studies, the
Hill coefficient for Na\(^+\) binding was generally taken to be around 2, whereas the estimates we obtained, consistent with those previously reported for NaP\(_i\)-2 (Busch et al., 1994), indicated a Hill coefficient significantly >2. These discrepancies might be due to inherent differences between the cloned transporter expressed in *Xenopus* oocytes and the native form in BBMVs, as well as possible contamination from type I Na\(^+\)/P\(_i\) cotransporters in the latter case. We previously reported (Forster et al., 1997a) a Hill coefficient for apparent Na\(^+\) binding significantly <2 for the flounder type II isoform (NaP\(_i\)-5) even though the \(K_m^{\text{Na}}\) was close to that for NaP\(_i\)-2. This may reflect real differences in the steady state kinetics of these two isoforms: for example, the larger Hill coefficient for NaP\(_i\)-2 might indicate a greater degree of cooperativity for Na\(^+\) binding (Weiss, 1997). However, a rigorous determination of the stoichiometry can only be undertaken by measuring the substrate uptake and electrogenic properties on the same oocyte (e.g., Eskandari et al., 1997). Moreover, the use of the Hill equation to infer a stoichiometry of the substrate binding should be approached with caution (Weiss, 1997) and the finding of an apparent 3:1 stoichiometry in the case of NaP\(_i\)-2, although attractive, should be conservatively taken as a lower limit.

**Order of substrate binding.** Two limitations of the intact oocyte preparation for studying cotransport function restrict the information obtained from steady state dose-response measurements: (a) there is no direct control of trans substrate concentrations, and (b) the normal osmolality of \(\sim 200\) mosM places an upper limit on the usable substrate concentrations, particularly in the case of Na\(^+\), where we were only able to measure the kinetics just above the predicted \(K_m^{\text{Na}}\). Given these limitations, we can nevertheless draw tentative conclusions about the order of substrate binding on the cis face. For P\(_i\) as the variable substrate, both “V” and “K” kinetics are found, where V kinetics refers to a maximum transport rate dependency on the fixed substrate and K kinetics refers to an apparent affinity constant dependency on the fixed substrate (e.g., Stein, 1990). This is indicated here by the dependency of both \(I_{\text{max}}\) and \(K_m^{\text{P}}\) on Na\(^+\). With K kinetics alone, random binding schemes can most likely be excluded, such as proposed by Béliveau and Strévey (1988) for Na\(^+\)/P\(_i\) cotransport in BBMVs. Furthermore, having V kinetics for the P\(_i\) dose dependency would be consistent with either P\(_i\) or Na\(^+\) being the last substrate to bind before translocation. On the other hand, although the maximum Na\(^+\) was \(120\) mM, our fits to the Na\(^+\) dose-dependency data suggest that only K kinetics are involved in the apparent Na\(^+\) binding. Taken together, this behavior would indicate that Na\(^+\) is the last substrate to bind. Yet, based on the steady state data alone, we cannot exclude the possibility that an additional Na\(^+\) binding step precedes P\(_i\) binding, as has been proposed for the Na\(^+\)/glucose cotransporter by Restrepo and Kimmich (1985), and Bennett and Kimmich (1992). Our finding of a Na\(^+\)-dependent slippage component, which was directly related to the expression level of functional NaP\(_i\)-2, also suggested that Na\(^+\) can interact with NaP\(_i\)-2 in the absence of P\(_i\) (see also Béliveau and Strévey, 1988).

In summary, the steady state data would be consistent with an ordered scheme for substrate binding on the cis face as seen in Scheme I, where \(C_o\) represents the unloaded carrier oriented towards the cis face. Based on the stoichiometry of the slippage component, we assumed a stoichiometry of 1:1 for the first Na\(^+\) binding reaction and that the second Na\(^+\) binding step involved two Na\(^+\) ions to account for the Na\(^+\) stoichiometry estimate of 3:1.

\[
C_o \leftrightarrow C_oNa \leftrightarrow C_oNaP_i \leftrightarrow C_oNaP_iNa^2 \]

\[\downarrow \text{slippage} \quad \downarrow \text{cotransport} \]

(Scheme I)

**Voltage dependence of substrate binding.** The apparent affinity constants for both substrates increased with membrane depolarization, but, as shown in Table III, the relative voltage sensitivity of \(K_m\) was itself dependent on the concentration of the fixed substrate. For example, for V in the range \(-100\) to 0 mV, the apparent P\(_i\) binding at 96 mM Na\(^+\) increased by 60%, whereas at 50 mM Na\(^+\) it increased more than threefold. In contrast, the relative voltage dependence of \(K_m^{\text{Na}}\) showed <50% increase over a 10-fold range of P\(_i\). These data suggest that the apparent binding of Na\(^+\), rather than P\(_i\), is a determinant of voltage dependence. This conclusion should be treated with caution since in any analytical expression for the steady state transport expressed in the form of Eq. 1, the apparent \(K_m\) will be a function of all the rate constants, including the unloaded carrier. Therefore, the voltage dependence of the apparent affinity for any substrate does not necessarily reflect the voltage dependence of the true affinity constant (Restrepo and Kimmich, 1985; Bennett and Kimmich, 1996).

**The effect of slippage on steady state kinetics.** The relatively high endogenous current in typical oocytes, compared with the P\(_i\)-induced component, necessitated subtraction of the endogenous background current from the total P\(_i\)-induced current under the assumptions that (a) the background current is only due to endogenous effects, and (b) it is P\(_i\) insensitive. Our finding of a Na\(^+\)-dependent slippage component similar to that first reported by Umbach et al. (1990) for the Na\(^+\)/glucose transporter, SGLT1, means that the validity of these assumptions must be reevaluated. The presence of slip-
page could lead to errors in interpreting steady state kinetic data, particularly at saturating P. If this component were suppressed in the presence of P due to a faster rate constant for the P binding step as P increases, an underestimate of the true coupled current by ~10% at saturating P would result, since both the endogenous component and a NaP-2-related component are subtracted from the test response.

**Pre-Steady State Kinetics**

Pre–steady state relaxations are a common feature of cation-coupled cotransporters that exhibit electroneutrality, being first reported by Birnir et al. (1990) for the cloned Na+/glucose cotransporter (SGLT1). Subsequent detailed kinetic studies of the SGLT family (e.g., Parent et al., 1992a, 1992b; Loo et al., 1993; Mackenzie et al., 1996; Hazama et al., 1997) and studies of other Na+-coupled cotransporters (e.g., Mager et al., 1993; Hager et al., 1995; Wadiche et al., 1995; Eskandari et al., 1997; Forster et al., 1997a) have established this technique as an important tool for identification of partial reactions in the transport cycle. In the present case, we have demonstrated that oocytes expressing NaP-2 exhibit pre–steady state relaxations with properties qualitatively similar to those previously reported for the flounder isoform, NaP-5 (Forster et al., 1997a) despite a four- to fivefold lower expression.

**Transport turnover.** The slope of the I_p(-100-Q(-100)) relation can be used to estimate the apparent turnover of NaP-2 at ~100 mV, assuming that (a) the charge translocation is a single step having an apparent valence z, and (b) the same number of transporters contribute to I_p(-100) in the presence of saturating P, as contribute to translocatable charge (Q(-100)) in the absence of P. The transporter turnover, φ, is then given by:

$$\phi = \frac{I_p(-100)}{Q_{(-100)}}$$  \hspace{1cm} (3)

Taking z = 0.5 from the Boltzmann fit to the QV relation and I_p(-100)/Q_{(-100)} = 46 s⁻¹, Eq. 3 gives \(\phi = 23 \text{ s}^{-1}\) at −100 mV and 96 mM Na⁺. This is comparable with the estimates of \(\phi\) for other Na⁺-coupled transporters expressed in *Xenopus* oocytes under similar conditions, including NaP-5 (35 s⁻¹) (Forster et al., 1997a).

**Voltage-dependent steps.** Like NaP-5 (Forster et al., 1997a), in 0 mM P, reducing Na⁺ resulted in a negative shift of Vₐ₀·₅ for the QV distribution without significantly affecting the total charge. However, if the charge movement were solely due to an “ion-well” effect, a much larger change in the voltage dependence of the τ-V relation would be expected (Forster et al., 1997a). Moreover, in 0 mM external Na⁺, a relaxation was still observed that was only suppressed if both substrates were present at the maximum concentrations used (96 mM Na⁺, 3 mM P). These findings suggested that (a) the unloaded carrier itself contributes significantly to the pre–steady state relaxations, and (b) Na⁺ is able to bind first, as deduced from the steady state analysis.
Figure 10. Simulations predict voltage-dependent behavior of pre–steady state kinetics in the absence of external P_i. Values were assigned to the parameters associated with voltage-dependent partial reactions (shown shaded in Fig. 9: unloaded carrier, $6 \Leftrightarrow 1$; first Na$^+$ binding/debinding, $1 \Leftrightarrow 2$) to give a reasonable match to the measured τ-V and Q-V data under varying external Na$^+$. (A) Simulations of pre–steady state current showing the ON and OFF relaxations for α step from $-100$ to 0 mV for two external Na$^+$ concentrations (continuous lines, 100 mM; broken lines, 50 mM). The voltage step is assumed to occur instantaneously so that no account is taken of the speed of oocyte membrane charging. Note that because of the voltage dependence of $k_{21}$, the fast component in the relaxation appears more prominent in the ON than OFF traces for both Na$^+$, and this can lead to significant errors in estimating $Q_{ON}$ and $Q_{OFF}$ (see discussion). Rate constants for voltage dependent steps are (s$^{-1}$): $k_{61} = 60 \exp(-0.16 \text{ eV}/\text{K}T)$, $k_{16} = 120 \exp(0.24 \text{ eV}/\text{K}T)$, $k_{12} = 8,000 \text{ [Na]} \exp(-0.15 \text{ eV}/\text{K}T)$, $k_{21} = 2,000 \exp(0.15 \text{ eV}/\text{K}T)$, where [Na] = Na$^+$ concentration (Molar). The corresponding valences and asymmetry factors are: $z_{61} = 0.4$, $z_{12} = 0.5$, $\delta_{61} = 0.4$, $\delta_{12} = 0.5$. Ordinate scale is in electronic units (eu) s$^{-1}$, where 1 eu = $1.602 \times 10^{-19}$ C. (B) Simulated τ-V relations for two external Na$^+$ concentrations as in A. Bold curves represent the two nonzero time constants predicted from the eigenvalue solutions of the three-state model involving transitions $6 \Leftrightarrow 1$ and $1 \Leftrightarrow 2$ (continuous curves, 100 mM; broken curves, 50 mM). The faster component with τ < 1 ms would not be detected easily by curve fitting due to the oocyte charging transient. Light curves represent the voltage dependence of the reciprocal rate constants for the transitions indicated by the respective subscripts. Note that only $k_{12}$ is dependent on Na$^+$. (C) Simulated steady state Q-V relation for the same two Na$^+$ concentrations. The amount of charge in electronic units contributed by the two transitions is shown ($Q_{61}$, $Q_{12}$), together with the total charge ($Q$). For the simulation, the holding potential was set at $-1,000$ mV to obtain normalized relations. Fitting Eq. 2 to the Q-V data predicted $z = 0.5$ and a shift of $-16$ mV for a change in Na$^+$ from 100 to 50 mM. (D) Simulation of the effect of pH on pre–steady state kinetics. The unloaded carrier backward rate constant ($K_{16}$) is assumed to be decreased from 120 to 60 s$^{-1}$, resulting from an increase in external H$^+$ as indicated by measurements. The τ-V curves predict an increase of the slower relaxation for $V > 0$. Continuous lines represent τs (bold) and inverse rate constants (light) under normal (pH 7.4) conditions. Broken lines are for reduced pH conditions. (E) The corresponding steady state Q-V relations for the same change in rate constant $K_{16}$ showing the shift in the Q-V distribution towards depolarizing potentials (broken lines) resulting primarily from a shift in the steady state charge distribution of the unloaded carrier.
since, in the alternative binding scheme $C_i \leftrightarrow C_iP \leftrightarrow C_iPNa$, no Na$^+$-dependent influence on the steady state charge distribution would be expected with 0 mM $P_i$.

For $P_i$ as the variable substrate, pre–steady state relaxations were also suppressed in a dose-dependent manner, but fitting to a single Boltzmann function showed no significant shift in $V_{0.5}$ and only a weak voltage dependence of the apparent $K_m$ for charge suppression, similar to the weak voltage dependence of $K_m^{Pi}$ at 96 mM Na$^+$. In agreement with our steady state findings, these results suggested that the apparent $P_i$ binding did not directly contribute to charge movements; i.e., the $P_i$ binding site lies outside the transmembrane electric field.

A characteristic feature of type II Na$^+$/Pi cotransport is its dependence on external pH, whereby H$^+$ ions are thought to compete for occupancy of the Na$^+$ binding site, thereby increasing the apparent $K_m^{Na}$ (Amstutz et al., 1985). Our finding of a shift of $V_{0.5}$ for the Q-V curve towards depolarizing potentials and a positive shift in the peak of the $\tau$-V data does not accord with the simple notion of H$^+$ ions either competing for occupancy of the Na$^+$ binding site and/or reducing the effective Na$^+$ concentration as seen by the first Na$^+$ binding site (i.e., ion-well). One candidate mechanism could involve the interaction of H$^+$ ions with the empty carrier. This is supported by our observation that, at 0 mM Na$^+$, the relaxations were also suppressed for a change in pH from 7.4 to 6.2, and findings from a recent study of the flounder isoform NaPi-5, where higher resolution recordings were possible (Forster et al., 1997).

**An Ordered Kinetic Model for NaPi-2**

Fig. 9 depicts the state diagram for a model that can account for the kinetic properties we have deduced for NaPi-2. This scheme is derived from that proposed for the cloned Na$^+$/glucose cotransporter (SGLT1) by Parent et al. (1992b), but includes a second Na$^+$ binding step to account for the dependence of $I_{max}$ on Na$^+$ in the steady state. Restrepo and Kimmich (1985) and Bennett and Kimmich (1996) have proposed a similar scheme, which also includes a second Na$^+$ binding step, to describe Na$^+$/glucose cotransport characterized in LLC-PK1 epithelia cells. To simplify the model, we assumed the binding of the two Na$^+$ ions (step 3 $\Leftrightarrow$ 4) occurs with strong positive cooperativity to give an apparent simultaneous binding (Falk et al., 1998; Weiss, 1997). As for $P_i$ binding (step 2 $\Leftrightarrow$ 3), this is assumed to occur external to the transmembrane field, so that it does not contribute to pre–steady state charge movements with finite $P_i$. With the two voltage-dependent steps identified from the pre–steady state data (6 $\Leftrightarrow$ 1 and 1 $\Leftrightarrow$ 2), the model successfully accounts for a number of the steady state results we have obtained.

![Figure 11](image-url) Simulations of $P_i$-induced currents in the steady state, assuming zero trans conditions. Since the transition 6 $\Leftrightarrow$ 1 is the only translocation step involving net charge movement, the steady state current is proportional to $X_6 k_{6} - X_1 k_{1}$, where $X_6$ and $X_1$ are the occupancy of states 1 and 6, respectively. Voltage-dependent rate constants and parameters are given in Fig. 10. Additional rate constants for the kinetic scheme of Fig. 9 are (s$^{-1}$): $k_{3a} = 1,000$ [P$^i_1$], $k_{2a} = 100$, $k_{3a} = 500$ [Na$^+$]$^2$, $k_{4a} = 50$, $k_{5a} = k_{3a} = 25$, $k_{5a} = 10,000$, $k_{5a} = 0$, $k_{5a} = 10,000$, $k_{5a} = 0$, $k_{5a} = 2.5$, $k_{5a} = 2.5$, where [P$^i_1$] and [Na$^+$] are the concentrations of P$^i_1$ and Na$^+$, respectively (Molar). These were chosen to give reasonable predictions of the experimentally observed I-V relations. (A) $P_i$-induced currents in the steady state, normalized to $I_{max}$ at $-100$ mV. Dashed curve is the slippage component (simulated with $P_i = 0$ mM), light curves represent the total simulated $P_i$-induced steady state current and bold curves represent the steady state response with slippage component subtracted (equivalent to the $P_i$-induced response measured). Eq. 1 was fit to the data to obtain the apparent $K_m^{Pi}$ as a function of V for nominal 100 (■) and 50 (□) mM Na$^+$. The continuous lines are for visualization only. (B) Na$^+$ dose response. (Inset) Set of I-V curves for nominal Na$^+$ values indicated, and 1 mM nominal $P_i$, normalized to $I_{max}$ at $-100$ mV. Light curves represent the simulated total steady state current, bold curves have slippage component subtracted (equivalent to the $P_i$-induced response measured). Eq. 1 was fit to the data to derive the apparent $K_m^{Na}$ as a function of V for nominal 1 (■) and 0.1 (□) mM $P_i$. The continuous lines are for visualization only.
ber of the features observed in both the pre–steady state and steady state.

**Two pre–steady state components.** Fig. 10 A shows simulated pre–steady state relaxations induced by a voltage step for two nominal Na⁺ concentrations (50 and 100 mM) in the absence of Pi. The corresponding τ-V and Q-V curves are shown in Fig. 10, B and C, respectively. As predicted from a three-state model, the total relaxation comprises two components that are visible in the simulation. In practice, because the fast component has a τ comparable with that of the intrinsic oocyte charging, its detection would be difficult with biexponential fitting to the total relaxation. Moreover, the inherent low-pass filtering by the passive membrane would further distort this component (Forster and Greeff, 1992). We obtained evidence for the second component by subtraction of the transient under saturating conditions (3 mM Pi, 96 mM Na⁺), whereby we assumed that all endogenous relaxations were fully suppressed. Further characterization of the fast component would require the cut-open oocyte technique (e.g., Chen et al., 1996) to improve the recording bandwidth and rise time.

**Voltage dependence of τ-V and Q-V relations.** For a reduction in external Na⁺, our data suggested that the main ON τ became slightly faster. With the translocation of the empty carrier (6 ↔ 1) as the rate limiting voltage-dependent step and external Na⁺ debinding faster than binding (i.e., k₂₁ > k₁₂), the simulation also predicts a small downward shift in the τ-V curve as external Na⁺ is reduced. Moreover, the Na⁺-dependent shift of V₀.5 for the ON Q-V distribution is also predicted by the model. Note that as the steady state charge distributions of both components are influenced by external Na⁺ (Fig. 10 C), a shift in V₀.5 would still be expected even if the fast component were not detected by single exponential curve fitting.

**Charge imbalance in total relaxation.** Our inability to detect the faster component in the complete relaxation can account for the charge imbalance of the apparent ON and OFF charge estimated from single exponential curve fitting. The voltage dependence of the fast component means that for depolarizing (ON) steps from Vₜ = −100 mV, single exponential fits commencing after the capacitive transient will accurately detect the slower component. However, for the corresponding OFF step, the fitted record now includes part of the fast component, thus giving a larger apparent charge for the OFF transition. The good agreement between QON and QOFF, by integrating the charge remaining after subtraction of the relaxation with saturating Pi, also supports this interpretation.

**pH effects.** We found the observed changes in the τ-V and Q-V could be simulated by assuming that H⁺ ions interact with the unloaded transporter to cause a reduction of the rate constant K₁₆. The τ-V relation (Fig. 10 D) shows the observed slowing of the main relaxation for depolarized potentials, and the corresponding Q-V curve (Fig. 10 E) predicts a positive shift in the steady state charge distribution as we observed.

**Steady state behavior.** For the Pi dose dependency (Fig. 11 A, inset), the predicted I-V curves indicate rate limiting behavior at extremes of membrane voltage and saturation at high Pi, as observed experimentally. Furthermore, at hyperpolarizing potentials, the subtraction procedure used to obtain the apparent Pi-induced current results in more pronounced rate limiting behavior due to the subtraction of the slippage component present in the control (0 mM Pi) response. Fitting Eq. 1 to the simulated data gave a voltage-independent Hill coefficient ≈ 1 that was independent of Na⁺ (data not shown) and an apparent Kᵢ₅ (Fig. 11 A) that increased monotonically with depolarizing voltage and was sensitive to Na⁺. For the Na⁺ dose response, the simulated I-V curves (Fig. 11 B, inset) are also qualitatively similar to the experimental data whereby, after subtraction of the response at 0 mM Pi, the Hill coefficient predicted from fitting Eq. 1 to the simulated I-V data was 2.4 and the apparent Kᵢ₅ varied weakly with potential for a 10-fold change in Pi (Fig. 11 B).

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