Deciphering PiT transport kinetics and substrate specificity using electrophysiology and flux measurements

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PiT-1, and human PiT-2) were expressed in oocytes, but only limited kinetic characterizations were made. To address this shortcoming, we performed a detailed analysis of SLC20 transport function. Three SLC20 clones (Xenopus PiT-1, human PiT-1, and human PiT-2) were expressed in Xenopus oocytes. Each clone gave robust Na⁺-dependent ³²P uptake, but only Xenopus PiT-1 showed sufficient activity for complete kinetic characterization by using two-electrode voltage clamp and radiomucle uptake. Transport activity was also documented with Li⁺ substituted for Na⁺. The dependence of the PiT-induced current on Pi concentration was Michaelian, and the dependence on Na⁺ concentration indicated weak cooperativity. The dependence on external pH was unique: the apparent PiT affinity constant showed a minimum in the pH range 6.2–6.8 of ~0.05 mM and increased to ~0.2 mM at pH 5.0 and pH 8.0. Xenopus PiT-1 stoichiometry was determined by dual ²²Na-³²P uptake and suggested a 2:1 Na⁺:Pi stoichiometry. A correlation of ³²P uptake and net charge movement indicated one charge translocation per Pi. Changes in oocyte surface pH were consistent with processes, and it is a component of many biological structures.

- Pi plays a central role in cell metabolism as well as in normal and pathological calcification.
- The two known mammalian PiT members, PiT-1 and PiT-2, have a broad tissue distribution (3, 30, 33, 50). This suggests that they may play a housekeeping role for Pi homeostasis in cells, but other roles for PiT proteins are also emerging. For example, PiT-mediated Pi transport appears to play an important role in providing Pi for the formation of mineralized bone (8, 46, 62). Furthermore, PiT proteins have been strongly implicated in pathological calcification of vascular tissue in response to hyperphosphatemia (31, 34, 39). Recently it was shown (43) that the malaria parasite Plasmodium falciparum expresses a PiT protein that is essential for providing the parasite with Pi for growth.

Although PiT plays a central role in cell metabolism as well as in normal and pathological calcification, the PiT transporters that provide cells with Pi have not been well characterized. Most studies have relied on radionuclide uptake measurements, where lack of membrane-potential control makes interpretation of kinetic studies of electrogenic transport processes difficult. Data analysis is further complicated by the low transport rates attained when expressing mammalian PiT-1 and PiT-2 heterologously. This may explain why, apart from the initial reports by Kavanaugh et al. (32, 33), no further electrophysiological characterization has been done.

We reasoned that Xenopus oocytes might express a Xenopus protein better than mammalian ones and decided to express a Xenopus homolog (XIPiT-1) as well as human PiT (hPiT)-1 and hPiT-2 in Xenopus oocytes. The PiT transport attained with XIPiT-1 far exceeded that of either mammalian isoform, and therefore most of our kinetic characterization was done using XIPiT-1. Using electrophysiology, radiotracer flux, and surface pH measurements, we show that PiT transports two Na⁺ ions for each Pi, and that the preferred species is monovalent H₂PO₄⁻. PiT transport is affected by pH because of the dependence of the H₂PO₄⁻:HPO₄⁻ ratio on pH, and between pH 6.2 and 8.0 there is no effect of H⁺ per se on PiT function. On the basis of the kinetics of substrate interdependence, we propose an ordered binding scheme of Na⁺:H₂PO₄⁻:Na⁺. Significantly, in contrast to type II Na⁺:Pi cotransporters, the transport inhibitor phosphonoformic acid did not inhibit PiT-1 or PiT-2 activity.

Na⁺:Pi cotransport; two-electrode voltage clamp; surface pH electrode; SLC20; retroviral receptor

INORGANIC PHOSPHATE (Pi) is fundamental to many metabolic processes, and it is a component of many biological structures. As a negatively charged anion, Pi has to be actively transported into the cell via an active transport process. In mammals, this task is carried out by two unrelated Na⁺:Pi cotransporter families. The well-characterized type II Na⁺:Pi cotransporter family (SLC34) has been shown to be instrumental for Pi absorption and reabsorption at the apical membrane of many epithelia (20, 25, 28, 29, 40). The type III Na⁺:Pi or PiT cotransporters (SLC20) were initially identified as retroviral receptors and later shown to be Na⁺:Pi cotransporters (33, 41, 60). PiT proteins are present in all kingdoms and use either the transmembrane Na⁺ (animals, fungi) or H⁺ (plants, bacteria) gradients to drive PiT transport.

The two known mammalian PiT members, PiT-1 and PiT-2, have a broad tissue distribution (3, 30, 33, 50). This suggests that they may play a housekeeping role for Pi homeostasis in cells, but other roles for PiT proteins are also emerging. For example, PiT-mediated Pi transport appears to play an important role in providing Pi for the formation of mineralized bone (8, 46, 62). Furthermore, PiT proteins have been strongly implicated in pathological calcification of vascular tissue in response to hyperphosphatemia (31, 34, 39). Recently it was shown (43) that the malaria parasite Plasmodium falciparum expresses a PiT protein that is essential for providing the parasite with Pi for growth.
report herein both complement and extend those of Ref. 53 and previous tracer-flux studies.

**MATERIALS AND METHODS**

**Molecular Biology and Oocyte Expression**

cDNAs encoding hPiT-1, XIPT-1, and hPiT-2 from the German Resource Center for Genome Research (RZPD) were subcloned into a KSM expression vector (56) to improve expression in *Xenopus laevis* oocytes. In some experiments, we used a type II Na\(^+\)-P\(_i\) transporter cloned from flounder (RZPD-1b) for comparison. The plasmids were linearized and were used as a template for the synthesis of capped cRNA by using the mMESSAGE mMACHINE T3 kit (Ambion).

Stage V–VI defolliculated oocytes from *X. laevis* were isolated and maintained as described previously (57). Oocytes were injected with 50 nl of cRNA (50 ng). Control oocytes were either injected with 50 nl of water or not injected. Oocytes were incubated at 16\(^\circ\)C in modified Barth’s solution, containing (in mM) 88 NaCl, 1 KCl, 0.41 CaCl\(_2\), 0.82 MgSO\(_4\), 2.5 NaHCO\(_3\), 2 Ca(NO\(_3\))\(_2\), and 7.5 HEPES, pH 7.4, adjusted with Tris. The solution was supplemented with 5 mg/l doxycycline.

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 and Solutions**

The solution compositions were as follows. Control superfusate (ND100) contained (in mM) 100 NaCl, 2 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), and 10 HEPES adjusted to pH 7.4 using Tris, unless otherwise stated (different pHs, different concentration of Ca\(^{2+}\) or Mg\(^{2+}\)). For pH ≤ 6.2, MES was substituted for HEPES. Na\(^+\)-free superfusate composition was as for ND100 with isosmotic substitution of choline chloride for NaCl (ND0) or LiCl (LD100). Solutions with intermediate Na\(^+\) concentrations were prepared by mixing ND0 and ND100 in appropriate proportions. For substrate test solutions, P\(_i\) was added from 1 M K\(_2\)HPO\(_4\) and KH\(_2\)PO\(_4\) stocks premixed to give the required pH. PFA was added from 100 mM stock; arsenate and sulfate were added from 1 M stocks.

**Radiotracer Uptake**

A group of oocytes (7–10 oocytes/group) was first allowed to equilibrate in solution without tracer. After aspiration of this solution, we added 100 nl uptake solution containing radiotracers ([\(^{32}\)P], alone or both [\(^{32}\)P] and \(^{22}\)Na). The uptake was allowed to proceed for 15–20 min before it was stopped by washing the oocytes four times with 4 ml ice-cold ND0 solution containing 0.5 mM cold P\(_i\), uptake of \(^{32}\)P alone was carried out by using ND100 solution and 1 mM cold P\(_i\), to which \(^{32}\)P (specific activity 10–20 mCi/mmole P\(_i\)) was added. Simultaneous uptake of \(^{32}\)P, and \(^{22}\)Na was done in ND50 solution (50 mM NaCl; pH 6.2 or pH 7.4) with 2 mM cold P\(_i\). These concentrations of cold P\(_i\) and Na\(^+\) were used to balance the specific activities of the two radionuclides. \(^{32}\)P was added to obtain 7 mCi/mmole P\(_i\), and \(^{22}\)Na was added to obtain 1.4 mCi/mmole Na\(^+\). After being washed, oocytes were placed individually in a scintillation vial and lysed in 250 \(\mu\)l 10% SDS. \(^{32}\)P activities of individual oocytes were counted by using a Packard Tri-Carb 2900TR scintillation counter. To separate the counts of \(^{32}\)P and \(^{22}\)Na, we programmed the counter for dual DPM assay with quench curves. Net P\(_i\) and Na\(^+\) uptake for each individual oocyte was plotted, and linear regression analysis was performed to obtain the Na\(^+\)-P\(_i\) transport stoichiometry.

**Electrophysiology**

Standard two-electrode voltage-clamp hardware was used [GeneClamp, Model 500 (Molecular Devices) or a laboratory-built clamp (17)]. Clamp hardware was controlled and data were acquired by using pClamp 8 software (Molecular Devices), which also controlled valves for solution switching. At the onset of each experiment, the oocyte was clamped to a holding potential (\(V_h\)) of −50 mV and was superfused with ND100 solution. To measure P\(_i\)-induced currents (\(I_{P_i}\)), the superfusate was switched to one containing P\(_i\) and the change in the holding current was monitored. When the current had reached a steady state, the perfusate was switched back and washout of P\(_i\) was monitored by observing the return of holding current to baseline.

**Rundown of Electrogenic Activity and Estimation of Membrane Capacitance**

Repeated application of P\(_i\) to voltage-clamped oocytes often resulted in a progressive loss of activity, which in severe cases amounted to >50% loss by the end of an experiment (see Fig. 2A). To take account of rundown in those experiments where repeated P\(_i\) applications induced a reduction in \(I_{P_i}\), successive test applications were bracketed with control substrate applications (1 mM P\(_i\) in ND100). These were normalized to the response at \(t = 0\) and were fitted with a single exponential that satisfactorily described the time course of rundown. The test values were then rescaled by a factor derived from the fit. To obtain a mechanistic insight into the rundown of \(I_{P_i}\), we correlated changes in the oocyte membrane capacitance (\(C_m\), indicative of changes in membrane area), with the decrease in \(I_{P_i}\) documented with repeated exposure of P\(_i\), \(C_m\) was determined by measuring the current transient in response to voltage steps from −50 mV to −60 mV and to −40 mV, integrating the capacitive transients and calculating the mean from the magnitude of the two charge estimates (23).

**Measurement of Apparent P\(_i\) and Na\(^+\) Affinities**

The oocyte was voltage clamped to \(V_h\) = −50 mV, and the holding current was continuously recorded. To measure \(I_{P_i}\), the superfusate was switched to one containing P\(_i\) and change in the holding current was monitored. When the current had reached a steady state, the superfusate was switched back and washout of P\(_i\) was monitored by observing the return of holding current to baseline. When \(I_{P_i}\) was recorded for another Na\(^+\) concentration or pH, the holding current was first allowed to stabilize at the new reference solution before being switched to one containing P\(_i\). To control for current rundown, each P\(_i\) test pulse was bracketed by a control (1 mM P\(_i\)) application, which was used to correct the measurements. To determine the apparent affinity constant for P\(_i\) (\(K_{P_i}^\text{app}\)), \(I_{P_i}\) was measured by using different P\(_i\) concentrations while keeping the Na\(^+\) concentration constant. For determining the apparent affinity constant for Na\(^+\) (\(K_{Na}^\text{app}\)), the oocyte was first perfused with a specific concentration of Na\(^+\) before being switched to one containing P\(_i\), (i.e., the P\(_i\) concentration was kept constant throughout the experiment). Estimates of \(K_{P_i}^\text{app}\) and \(K_{Na}^\text{app}\) were obtained by fitting data with the modified Hill equation given by

\[
I_{P_i} = I_{P_i}^{\text{max}} [S]_{\text{app}}/(K_{P_i}^\text{app} + [S]_{\text{app}}) + I_{\text{OFF}}
\]

where \(I_{P_i}^{\text{max}}\) is the maximum current attainable, \(I_{\text{OFF}}\) is a variable offset (see below), [S] is the variable substrate concentration, and \(n_{h}\) is the Hill coefficient.

The voltage dependence of \(I_{P_i}\) was measured by applying potentials from −160 or −140 mV to +40 mV and subtracting the current records for the same potential in the presence and absence of P\(_i\), as described previously (17, 55). To account for the differences in expression levels between individual oocytes, data obtained from each oocyte were normalized to \(I_{P_i}\) recorded at −100 mV with 100 mM Na\(^+\) and 1 mM P\(_i\) in the bath at pH 7.4 before the data was fitted with Eq. 1. The offset was included to account for the leak current that we have documented in NaPi-IIa-expressing oocytes in the absence of P\(_i\), but that is blocked by P\(_i\), with an unknown affinity (12, 55).
Pre-Steady-State Current Measurements

Pre-steady-state currents were recorded by applying voltage steps from \( V_h = -60 \, \text{mV} \) to test potentials. To improve the signal resolution without distorting the current during the membrane-charging phase when exogenous charge movements might occur, the endogenous capacitive transient was partially suppressed by using a capacitive transient simulator.

Simultaneous Voltage Clamp and \(^{32}\text{P}\) Uptake

These experiments were carried out as previously described (2, 55). Briefly, an oocyte was placed in a superfusion chamber and the membrane voltage was clamped to \(-80 \, \text{mV}\). After the holding current had stabilized, the superfusate was switched to ND100 (pH 7.4 or pH 6.2) to ND100 solution (with the same pH) containing 1 mM cold \( \text{P}\) and \(^{32}\text{P}\), at a specific activity of 5 mCi/mmol Pi. After \(~5\) min, the perfusate was switched back to ND100 solution, and washout of \( \text{P}\) was monitored by following the return of the current to baseline. The oocyte was then removed from the chamber and lysed in 10% SDS, and oocyte radioactivity was counted by using a scintillation counter.

The net charge (Q) translocated by the transporter was calculated by first subtracting the baseline holding current and then by integrating the \( I_{V_h} \). Net \( \text{P}\) uptake and Q were plotted for each individual oocyte, and linear regression analysis was used to obtain the P:Q translocation ratio.

Surface pH Measurements

\( \text{pH}\)-sensitive microelectrodes of the liquid-membrane type (1) were manufactured as previously described (19). For surface pH measurements, we used electrodes with a large tip diameter (~20 \( \mu\)m) that were fire polished to obtain a smooth surface. The \( \text{pH}\) electrode potential was measured by using a laboratory-built unity-gain electrometer amplifier. The signal from the \( \text{pH}\) electrode was electronically subtracted from that of the \( V_m \) electrode of the two-electrode voltage clamp. Because the \( V_m \) electrode was intracellular and the \( \text{pH}\) electrode extracellular, the \( V_m \) (intracellular – bath) was then subtracted from the signal to obtain extracellular \( \text{pH}\). The \( \text{pH}\) electrodes were calibrated by using a two-point calibration (pH 8.0 and 6.0).

Continuous current and \( \text{pH} \) recordings were obtained from voltage-clamped (\( V_h = -10 \, \text{mV}, -50 \, \text{mV}, \) or \(-100 \, \text{mV}\)) control oocytes or oocytes expressing \( \text{XIPIT-1} \) or flounder NaPi-IIb. In these experiments, the buffering power of the ND100 solution was decreased by reducing the amount of HEPES from 10 to 2 mM. The response of the \( \text{pH}\) electrode to application of 1 mM \( \text{P}\) was recorded with the \( \text{pH}\) electrode either in the bath or pressed firmly against the oocyte surface. Because applying \( \text{P}\), with the \( \text{pH}\) electrode in the bath caused a deflection in the \( \text{pH}\) signal of 5–8 mV, possibly due to an unspecific interaction of the \( \text{P}\) ion with the liquid membrane, we subtracted the \( \text{P}\)-induced deflection obtained with the electrode in the bath from the one obtained with the electrode pressed against the oocyte surface.

RESULTS

Expression of Type III Na-\( \text{P}\), Cotransporter Isoforms in Xenopus Oocytes and Basic Transport Characteristics

We first investigated the \( \text{P}\), transport capabilities of the different clones by performing \(^{32}\text{P}\), uptake in 100 mM Na, 100 mM choline, and 100 mM lithium. Figure 1A shows \( \text{P}\), uptake measured in oocytes expressing hPiT-1, XIPIT-1, and hPiT-2 and in uninjected oocytes. \( \text{P}\), uptake was much higher in oocytes expressing XIPIT-1 than in either human isoform. \( \text{P}\), uptake was highest in the presence of Na\(^+\) for all isoforms, but we also observed \( \text{P}\), uptake in choline and Li\(^+\) that was significantly larger than in control oocytes (except for hPiT-1 in choline solution).

Next, we investigated the electrogenic response of hPiT-1, XIPIT-1, and hPiT-2 to 1 mM \( \text{P}\), in ND100 solution. First we recorded a continuous current response to 1 mM \( \text{P}\), by using \( V_h = -50 \, \text{mV} \) (Fig. 1B). Application of 1 mM \( \text{P}\), induced an inward current, which indicated transport of positive charge into the cell. The magnitude of the \( I_{V_h} \) was highest in oocytes expressing XIPIT-1, intermediate in hPiT-2, and lowest in hPiT-1, which paralleled the \( \text{P}\), uptake results documented in Fig. 1A. It is conceivable that the difference in \(^{32}\text{P}\), transport and \( I_{V_h} \) results from different expression levels of the three isoforms; however, because the turnover rate and the number of active transporters in the membrane are not known for any of the isoforms, this remains speculative. Uninjected oocytes from the same batch did not show \( \text{P}\),-induced inward current (data not shown).

The voltage dependence of \( I_{V_h} \) in XIPIT-1 and hPiT-2 is shown in Fig. 1C. Again, XIPIT-1-expressing oocytes showed a higher \( I_{V_h} \) than the human isoform, but the voltage dependence of \( I_{V_h} \) was similar for the two clones. We were unable to...
determine with confidence the voltage dependence of $I_{Pi}$ in oocytes expressing hPiT-1 because of the small currents.

Rundown of $I_{Pi}$. On repeated and continuous $P_i$ application, we observed that $I_{Pi}$ in XIPiT-1-expressing oocytes decreased. This phenomenon was particularly severe in oocytes giving large $I_{Pi}$ ($\sim -100 \text{ nA at } V_m = -50 \text{ mV}$). Figure 2A shows a current recording from a representative oocyte where we applied 1 mM $P_i$ for 20 s at 1-min intervals. Such a loss of activity could result from accumulation of substrate of the trans side of the membrane, resulting in inhibition of the forward transport rate. Alternatively, it could result from a reduction in the number of active cotransporters in the membrane due to endocytosis (23). To investigate the latter hypothesis, we investigated whether changes in the oocyte whole cell capacitance accompanied the decrease in $I_{Pi}$. The capacitance was taken as a measure of the oocyte membrane area, and a decrease in capacitance ($C_m$) would indicate that endocytosis had occurred. Figure 2B shows $I_{Pi}$ plotted against the change in $C_m$ ($\Delta C_m$) induced by repeated $P_i$ applications. The decrease in $I_{Pi}$ was accompanied by a decrease in $C_m$ that strongly suggested that endocytosis of transporters was induced by repeated $P_i$ exposure. However, the correlation between the decrease in $I_{Pi}$ and $\Delta C_m$ was not linear, and therefore other mechanisms must also play a role.1

$P_i$ Dependence of XIPiT-1 at pH 7.4

To investigate the electrogenic response of XIPiT-1 to $P_i$ in more detail, we determined the $P_i$ dependence at different $V_m$. Pooled current-voltage (I-V) data are shown in Fig. 3A for $P_i$ varying from 0.01 mM to 1 mM with 100 mM Na$^+$, pH 7.4. At the lowest $P_i$ concentration tested (0.01 mM), the slope of the I-V data was positive and reversed direction in the range $-30 \text{ mV to } -50 \text{ mV}$. The response to 0.01 mM $P_i$ varied between batches of oocytes and suggested that a $P_i$-dependent leak component was also present, as previously reported for the type II Na$^+$-P$_i$ cotransporter (17). For all other $P_i$ concentrations, the currents did not reverse, even up to $+60 \text{ mV}$, for those oocytes where endogenous activating currents were judged to be negligible (data not shown). By transposing the I-V data, we obtained the $P_i$ dependence at each test potential (Fig. 3B). The data were well described analytically by fitting them with a form of the Michaelis-Menten function (Eq. 1, with $n_H = 1$) for test potentials $\pm 0 \text{ mV}$. We accounted for the putative $P_i$-inhibitable leak component by including a variable offset in the fit function (see Measurement of Apparent $P_i$ and Na$^+$ Affinities) as previously described (12, 55). For potentials $>0 \text{ mV}$, the $P_i$ dependence was prone to contamination by endogenous activating currents, which precluded analysis in this region. The Michaelian behavior suggested that there was one $P_i$ interaction site for each XIPiT-1 transporter. These fits yielded an estimate for the apparent $K_{P_i}^{0.5}$ that was independent of the test potential (Fig. 3F), and the predicted maximum electrogenic activity ($I_{P_i}^\max$) showed a curvilinear voltage dependence with no evidence of rate-limiting behavior at the hyperpolarizing limit (Fig. 3E). The lack of voltage dependence of $K_{P_i}^{0.5}$ with 100 mM Na$^+$ superfusion suggested that $P_i$ did not interact with the transmembrane electric field.

To determine the nature of the substrate interaction (i.e., ordered vs. random binding), we repeated this assay by superfusing oocytes in 50 and 25 mM Na$^+$. To aid comparison, we normalized the data to the response to 1 mM $P_i$ at $-100 \text{ mV}$ and 100 mM Na$^+$. Reducing external Na$^+$ led to a concomitant reduction in currents at all potentials, as illustrated for 25 mM Na$^+$ superfusion (Fig. 3C), and we also observed a $P_i$ dependence. Like the behavior in 100 mM Na$^+$, these data were well described by fitting with Eq. 1 to yield estimates for $K_{P_i}^{0.5}$ and $I_{P_i}^\max$. The predicted $K_{P_i}^{0.5}$ at 50 mM Na$^+$ exceeded $K_{P_i}^{0.5}$ at 100 mM Na$^+$ slightly and deviated at depolarizing potentials. This trend was even more obvious for 25 mM Na$^+$; at $-100 \text{ mV}$, $K_{P_i}^{0.5}$ doubled and the voltage dependence was clearly evident in the depolarizing direction (Fig. 3F). The behavior suggested that voltage-dependent transitions in the transport cycle were rate determining and dependent on Na$^+$ ion availability. The maximum $P_i$-induced current ($I_{P_i}^\max$) obtained from the fits was also dependent on external Na$^+$ (Fig. 3E), which implied that the interaction of Na$^+$ and $P_i$ with XIPiT-1 was ordered (45). Moreover, if we assume that $I_{Pi}$ is a measure of the number of carrier proteins with fully bound substrate the dependence of $I_{Pi}^\max$ on Na$^+$ would be consistent with Na$^+$ ions being the last substrate to bind before translocation (45).

Na$^+$ Dependence of XIPiT-1 at pH 7.4

To investigate the dependence of $I_{Pi}$ on external Na$^+$, we determined the I-V relationship for $I_{Pi}$ by varying the external Na$^+$ from 0 to 100 mM, with $P_i = 1 \text{ mM}$ (Fig. 4A). As expected, at all test potentials, $I_{Pi}$ decreased in a concentration-dependent manner with decreasing external Na$^+$. I-V data were pooled and normalized to $I_{Pi}$ induced by 1 mM $P_i$ at $-100 \text{ mV}$, to take account of different expression levels. As for the $P_i$-dependence assays, for $V_m > 0 \text{ mV}$, the data were less reliable due to contamination from endogenous activating currents, and analysis was only performed for $V \leq 0 \text{ mV}$. The data were transposed into a Na$^+$-dependence relationship that showed obvious saturation at all test potentials. Together with our finding of a saturable $P_i$ dependence of the electrogenic response (Fig. 3B), the Na$^+$ dependence provided complementary evidence for carrier-type behavior for XIPiT-1 with respect to Na$^+$ as the variable substrate. The Na$^+$-dependence data were well described by fitting with the modified Hill equation (Eq. 1; Fig. 4B). With all four fit parameters unconstrained, the fits predicted a $n_H$ close to unity. An F-test that compared the fits for the unconstrained case ($n_H$ as a free parameter) with the Michaelian model ($n_H = 1$) indicated that there was no statistical difference ($P > 0.3$) between the two models for all test potentials. All subsequent fitting for determination of the apparent affinity constant for Na$^+$ ($K_{Na}^{0.5}$) and maximum $P_i$-inducible current ($I_{P_i}^\max$) was therefore constrained with $n_H = 1$ to reduce the uncertainty in estimating these parameters. If we assume that more than one Na$^+$ ion is translocated per transport cycle, which would be the most straightforward explanation for the observed electrogenicity, the Michaelian behavior of the Na$^+$ dependence suggests that there was little cooperativity for the interaction of Na$^+$ ions with the transporter.

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1 Rundown of response has also been reported for the SLC5A8 Na$^+$-monocarboxylate cotransporter (9), and these authors suggested that it resulted from trans inhibition, because it was not observed at low substrate concentrations. We have also documented rundown behavior for oocytes expressing the flounder NaPi-Ib, where a decrease in $C_m$ was also found (I. C. Forster, unpublished data). The phenomenon also appeared to depend on the batch of oocytes and, in the case of PiTs, suggested that internalization of transporters occurs as a result of Pi-activated oocyte-signaling pathways.
We obtained further insight into the nature of substrate interactions by studying the Na\(^+\)/H\(^+\) dependence at Pi concentrations close to the predicted apparent K\(_{0.5}\). For 0.3 mM total Pi (data not shown) and 0.1 mM total Pi (Fig. 4C), we also observed a monotonic dependence of \(I_{\text{Pi}}\) on Na\(^+\). To compare the different Pi conditions, we normalized each data point to \(I_{\text{Pi}}\) induced by 1 mM Pi at \(-100\) mV and superfusion in 100 mM Na\(^+\). After transposition of these data, saturation with respect to Na\(^+\) was also evident, and the data were similarly well described by a Michaelian relationship (Fig. 4D) for test potentials \(-40\) mV. The normalized \(I_{\text{Pi}}^{\text{max}}\) was obviously dependent on Pi (Fig. 4E). The apparent affinity for Na\(^+\) was also voltage dependent, and \(K_{0.5}\) increased with membrane hyperpolarization (Fig. 4F). There was no statistical difference for \(K_{0.5}\) with Pi = 0.3 mM and Pi = 0.1 mM, \(K_{0.5}\) increased markedly in the depolarizing direction. At hyperpolarizing potentials, the \(K_{0.5}\) data suggested that there was an asymptotic limit for this parameter that was independent of Pi and \(V_m\).

Transport Stoichiometry of XIPiT-1

Simultaneous voltage clamp and uptake of \(^{32}\)Pi. We measured the Q transferred for each Pi molecule transported by performing simultaneous voltage clamp and \(^{32}\)Pi uptake measurements in oocytes expressing XIPiT-1. Uninjected oocytes served as controls. This assay was performed at two pH values (6.2 and 7.4) to determine whether the transporter has a preference for mono- or divalent Pi. For a \(pK_a\) of 6.8, the ratio \(\text{H}_2\text{PO}_4^-:\text{HPO}_4^{2-}\) at pH 6.2 is 4:1, whereas at pH 7.4 it is 1:4. If

Fig. 3. Pi dependence of XIPiT-1 at pH 7.4. A: current-voltage (I-V) data for \(I_{\text{Pi}}\) with total Pi as the variable substrate and Na\(^+\) fixed at 100 mM, normalized to the response to 1 mM Pi at \(-100\) mV in 100 mM Na\(^+\) (n = 7). Data points are joined for visualization only. B: transformation of data in A to show Pi dependence at each test potential. Continuous lines are fits using Eq. 1. C: I-V data obtained in 25 mM Na\(^+\) and normalized as in A (n = 9). D: transformation of data in C fitted with Eq. 1. E: voltage dependence of maximum Pi-induced current (\(I_{\text{Pi}}^{\text{max}}\)) reported by fitting Eq. 1 to the Pi dependencies for the 3 Na\(^+\) concentrations indicated. F: voltage dependence of apparent affinity constant of Pi (\(K_{0.5}\)) reported by curve fitting for 3 Na\(^+\) concentrations indicated. Error bars in E and F indicate SE of fit. For Na\(^+\) = 50 mM, data were pooled from n = 5 oocytes.
both species were transported with similar efficiencies, we would expect the Q:Pi ratio to change with different pH (assuming that the number of Na\(^+\) ions transported per Pi remain unchanged). Figure 5A shows an original current trace of and oocyte expressing XlPiT-1 and held at \(V_h = -80\) mV. The oocyte was initially superfused with ND100 solution; then 1 mM Pi with \(^{32}\text{Pi}\) as a tracer was applied as indicated. After washout of Pi, the holding current was allowed to return to baseline before the oocyte was removed and lysed for scintillation counting to measure intracellular \(^{32}\text{Pi}\). The Q moved was calculated by integrating the area under the \(I_{\text{Pi}}\) trace. Figure 5B shows transferred Q plotted against the amount of transported Pi, for each individual oocyte. Linear regression analysis showed that the ratio of charge to Pi was close to unity for both pH values tested (0.93 ± 0.04 at pH 7.4 and 0.82 ± 0.03 at pH 6.2), indicating that one charge is moved for each transported Pi. In control oocytes exposed to the same experimental manipulation as oocytes expressing XlPiT-1, no \(I_{\text{Pi}}\) were observed and the amount of Pi taken up was minimal.

Dual uptake of \(^{32}\text{Pi}\) and \(^{22}\text{Na}\). Next we performed simultaneous uptake of \(^{32}\text{Pi}\) and \(^{22}\text{Na}\) at pH 6.2 and 7.4 to measure the Na:Pi transport stoichiometry. These experiments were per-

![Fig. 4. Na\(^+\) dependence of XlPiT-1 at pH 7.4. A: I-V data for \(I_{\text{Pi}}\) with Na\(^+\) as the variable substrate and Pi fixed at 1 mM (total), normalized to the response to 1 mM Pi, at \(-100\) mV in 100 mM Na\(^+\) (n = 7). Data points are joined for visualization only. B: transformation of the data in A to show Na\(^+\) dependence at each test potential. Continuous lines are fits using Eq. 1. C: I-V data obtained with 0.1 mM Pi (total) and normalized as in A (n = 6). D: transformation of the data in C and fitted with Eq. 1. E: voltage dependence of \(I_{\text{Pi}}\) reported by fitting Eq. 1 to the Na\(^+\) dependencies for the 3 Pi concentrations indicated. F: voltage dependence of apparent affinity constant of Na (\(K_{0.5,\text{Na}}\)) reported by curve fitting for 3 Pi concentrations indicated. Error bars in E and F indicate SE of fit. For Pi = 0.3 mM, data were pooled from n = 7 oocytes.

Fig. 5. Transport stoichiometry of XlPiT-1. A: original trace showing \(I_{\text{Pi}}\) in an oocyte expressing XlPiT-1 at a holding potential (\(V_h\)) of \(-80\) mV. Dashed line indicates baseline. B: transferred charge Q was obtained by integrating \(I_{\text{Pi}}\) from recordings similar to A and plotted as a function of Pi uptake (measured using \(^{32}\text{Pi}\)) into the same oocyte. The slope of Q:Pi was obtained by using linear regression and was 0.93 ± 0.04 for pH 7.4 and 0.82 ± 0.03 for pH 6.2. C: unidirectional Na\(^+\) uptake (measured using \(^{22}\text{Na}\)) was plotted against Pi uptake (measured using \(^{32}\text{Pi}\)) for each oocyte. The slope of Na:Pi was obtained by using linear regression and was 1.6 ± 0.1 for pH 7.4 and 1.7 ± 0.1 for pH 6.2. The regression lines were forced through the values measured for noninjected oocytes.
formed without voltage clamping the oocytes. We reduced the Na\(^+\) concentration in the solution to 50 mM and increased the P\(_i\) concentration to 2 mM to ensure that the energy spectra of the radioactive isotopes were well separated by the scintillation counter. The amount of Na\(^+\) and P\(_i\) taken up by each oocyte was calculated from the amount of radioactivity accumulated in each cell. Figure 5C shows a plot of Na\(^+\) uptake as a function of P\(_i\) uptake for each individual cell. Using linear regression analysis, we obtained a Na:P\(_i\) ratio of 1.6 ± 0.1 for pH 7.4 and 1.7 ± 0.1 for pH 6.2, suggesting that the Na:P\(_i\) stoichiometry is 2:1. However, because the measured Na:P\(_i\) ratio was not exactly an integer, it is possible that another substrate, for example H\(^+\), may substitute for Na\(^+\). However, because there was no difference between the slopes of the regression lines measured at pH 6.2 and 7.4, representing a 16-fold difference in H\(^+\) concentration, it seems unlikely that H\(^+\) would play a significant role as transported substrate.

Surface pH measurements. Finally, we made surface pH measurements to determine if XlPiT-1 preferentially transported monovalent or divalent P\(_i\). If the transporter were to remove monovalent H\(_2\)PO\(_4^-\) from the extracellular solution, we would expect an alkalinization to occur, because excess HPO\(_4^{2-}\) left behind would combine with H\(^+\) to form H\(_2\)PO\(_4^-\), thus causing an increase in pH. On the other hand, if divalent HPO\(_4^{2-}\) were the preferred species, we would expect to observe an acidification. Inside the oocyte, the pH changes would be reversed. However, we did not see any P\(_i\)-induced changes in intracellular pH in oocytes expressing NaPi-IIb (I. C. Forster, unpublished observations) or in XlPiT-1-expressing oocytes (data not shown). This is most likely due to the low transport rate accomplished when expressing these transporters in oocytes and the high buffering capacity of the oocyte [see Ref. 10].

To observe pH changes in the bath solutions due to XlPiT-1 transport activity, we fabricated pH-sensitive microelectrodes with a large tip area and fire-polished them to be very smooth, so that they did not damage the oocyte surface. Pressing the tip of such an electrode onto the top of an oocyte creates a microenvironment to which diffusion of substrate from the bulk medium is slowed down (but not blocked). This enables pH changes caused by P\(_i\) transport to be recorded, provided that the P\(_i\) removal rate by the transporter exceeds the H\(^+\) diffusion rate to or from the bulk medium. To magnify any pH change occurring, we reduced the buffering power of the ND100 solution from 7.2 to 1.4 mM/ΔpH by reducing the HEPES concentration from 10 to 2 mM.

Figure 6A shows surface pH recording from a control oocyte, an oocyte expressing XlPiT-1, and an oocyte expressing flounder NaPi-IIb, along with the corresponding current recordings. Application of 1 mM P\(_i\) caused a small deflection in the voltage signal from the pH electrode, which translated into an apparent decrease in pH of 0.02–0.05 pH units. We believe, however, that this may be an artifact caused by interaction of P\(_i\) ions with the liquid membrane electrode, because we observed no difference in pH between the two solutions with a glass electrode. When we repeated the P\(_i\) application with the pH electrode pressed against the surface of a control oocyte (“ON”) we observed an identical deflection in the signal from the pH electrode. However, when the pH electrode was pressed against the surface of an XlPiT-1-expressing oocyte, the direction of the pH change was reversed, showing that an alkalinization occurred on the surface of the oocyte. This would be consistent with transport of monovalent H\(_2\)PO\(_4^-\) by XlPiT-1. In contrast, when we pressed the pH electrode onto the surface of an oocyte expressing flounder NaPi-IIb, the magnitude of the pH deflection was increased. This acidification of the oocyte surface indicated that NaPi-IIb transports divalent HPO\(_4^{2-}\), which is in excellent agreement with previous studies (2, 22, 55) on the stoichiometry and substrate specificity of the type II Na\(^+\)-P\(_i\) cotransporter family.

Figure 6B summarizes surface pH changes recorded in control oocytes and in oocytes expressing XlPiT-1 or NaPi-IIb at three different potentials. The pH change was calculated from the difference in the P\(_i\)-induced change in pH observed with the pH electrode pressed onto the oocyte surface (ON) and with the pH electrode freely in the bath (OFF). No pH changes were observed on the surface of control oocytes at any V\(_m\). In contrast, we consistently observed alkalinization on the surface of XlPiT-1-expressing oocytes, whereas acidification was observed for NaPi-IIb. Both responses were statistically different.
from the values obtained in control oocytes, as determined by using two-way ANOVA. The magnitude of the pH change tended to increase with increasing hyperpolarization (which increases the transport rate); however, this trend did not reach statistical significance.

Role of metal-ion complexes. Bottger et al. (4) recently reported that $^{32}$P uptake into oocytes expressing hPiT-1 and hPiT-2 was reduced by $\sim 40$ and $\sim 42\%$, respectively, when Ca$^{2+}$ and Mg$^{2+}$ were omitted from the incubation medium. They suggested that divalent cations modulate the Pi-transport capacities of the proteins rather than Pi being transported as a metal complex, as has been reported for some bacterial Pi transporters (51, 52). We addressed the role of divalents by measuring the current induced by 1 mM Pi in oocytes expressing XlPiT-1. We found that $I_{\text{Pi}}$ was reduced by 29 $\pm$ 2$\%$ when both Ca$^{2+}$ and Mg$^{2+}$ were removed and by 15 $\pm$ 2$\%$ and 12 $\pm$ 1$\%$ when either Ca$^{2+}$ or Mg$^{2+}$ was removed, respectively ($n$ = 6). These values were significantly smaller than reported by Bottger et al. (4).

pH Dependence of P$_i$ Transport in XlPiT-1

To determine the pH dependence of P$_i$ transport by PiT-1, we measured the apparent affinity constant for P$_i$ ($K_{\text{Pi}}^{0.5}$) in XlPiT-1-expressing oocytes at seven pH values in the range 5.0–8.0. The $V_m$ was held at $-50$ mV, and deflections in $I_{\text{Pi}}$ induced by application of P$_i$, at different concentrations were monitored. The oocyte responses were normalized to $I_{\text{Pi}}$ obtained with 1 mM P$_i$, pH 7.4, to allow pooling of data from oocytes with different expression levels. We plotted $I_{\text{Pi}}$ as a function of P$_i$ concentration (Fig. 7A) and fitted the data with Eq. 1 ($n_H$ was constrained to 1). For clarity, only four pH conditions are shown. Figure 7B shows the fitted parameters $K_{\text{Pi}}^{0.5}$ (left axis) and $I_{\text{Pi}}^{\text{max}}$ (right axis) as a function of pH. The data show that $I_{\text{Pi}}^{\text{max}}$ is unaffected by a change in pH between 5 and 7.4. $I_{\text{Pi}}^{\text{max}}$ slightly decreased only when pH increased to 8.0. In contrast, large changes were documented for the apparent P$_i$ affinity. $K_{\text{Pi}}^{0.5}$ increased both when pH was lowered below pH 6.2 or increased above pH 6.8 (Fig. 7B). However, given a $P_{\text{K}}$ for H$_2$PO$_4^{-}$:HPO$_4^{2-}$ of 6.8, and because the preferred substrate for XlPiT-1 is H$_2$PO$_4^{-}$ (Fig. 6), it would appear that the increase in $K_{\text{Pi}}^{0.5}$ seen when pH was increased above pH 6.8 resulted from a reduced availability of monovalent H$_2$PO$_4^{-}$. Indeed, if we calculate the apparent affinity constant for H$_3$PO$_4$ ($K_{\text{Pi}}^{\text{HPP}}$) instead of for P$_i$ (Fig. 7B), we see essentially no change in $K_{\text{Pi}}^{\text{HPP}}$ for pH values $>$6.8.

To investigate whether protons alter the Na$^+$ dependence, for example by substituting for Na$^+$ or modulating the kinetics of Na$^+$ interaction, we repeated the Na$^+$-dependence assay at pH 6.2 and 8.0 for $V_m$ in the range from $-140$ mV to 0 mV by using 1 mM P$_i$. Na$^+$ dose dependencies were fitted with Eq. 1 as before to obtain estimates for $K_{\text{Na}}^{0.5}$ and $I_{\text{Na}}$. As shown in Fig. 7C, the normalized maximum attainable current showed the same voltage dependence for all pH values. Furthermore, for pH 7.4 and 8.0, we documented no significant alteration in $K_{\text{Na}}^{0.5}$; however, the Hill coefficient ($n_H$) tended to decrease when pH was increased above pH 6.8. As shown in Fig. 7D, the apparent Hill coefficient ($n_H$) decreased when pH increased from 5.0 to 8.0.

Fig. 7. pH dependence of P$_i$ transport by XlPiT-1. A: $I_{\text{Pi}}$ at $-50$ mV were determined at different concentrations of P$_i$ over the pH range 5.0–8.0. Data obtained from each oocyte were normalized to $I_{\text{Pi}}$ measured at pH 7.4, 1 mM P$_i$. Pooled data were fit with Eq. 1 (Hill coefficient ($n_H$) constrained to 1; continuous lines). For clarity, only data for 4 of the 7 different pH conditions are shown. B: fit parameters from data in A were plotted as a function of pH. Since $K_{\text{Pi}}^{0.5}$ concentration changes with pH, we calculated both $K_{\text{Pi}}^{0.5}$ and $K_{\text{Pi}}^{0.5}$, $n_H$. Left axis, $K_{\text{Pi}}^{0.5}$ and $K_{\text{Pi}}^{0.5}$; right axis, $I_{\text{Pi}}$. Asterisk indicates that value differs significantly ($P < 0.05$, ANOVA) from corresponding value at pH 7.4. No statistics were calculated for $K_{\text{Pi}}^{0.5}$. C: $I_{\text{Pi}}$ plotted as a function of voltage for 3 pH values. There was no statistical difference between points over range of voltages shown. $D$: $K_{\text{Na}}^{0.5}$ plotted as a function of voltage for 3 pH values. Data for pH 7.4 and 8.0 were not statistically different, but pH 6.2 data were significantly different. E: $n_H$ of Na$^+$ dependence fits plotted as a function of voltage for 3 pH values. Two-way ANOVA indicated that $n_H$ was significantly influenced by pH but not by voltage. In C, D, and E, data are shown as means ± SE; pH 6.2 ($n = 8$); pH 7.4 ($n = 10$); pH 8.0 ($n = 9$).
either the magnitude or voltage dependence of $K_{Na^+}$, whereas at pH 6.2, $K_{Na^+}$ showed little variation with voltage and the apparent affinity for $Na^+$ increased. This behavior reflects the interdependence of substrates in determining their apparent affinities because at pH 6.2, there is an increased availability of the preferred species, $H_2PO_4^-$. We also observed that at pH 8.0, fits to the $Na^+$-dependence data with $n_H$ as a free parameter were improved compared with those by fixing $n_H = 1$. This behavior suggested that at the higher pH the cooperativity of $Na^+$ interaction increased.

**Substrate Specificity**

Arsenate is structurally similar to $P$ and is a known substrate for members of the SLC34 family of $Na^+P$ cotransporters (7, 27). To investigate whether arsenate is also a substrate for the type III family, we performed $^{32}P$ uptake and electrophysiology assays. First, we performed $^{32}P$ uptake on un.injected oocytes and oocytes expressing hPiT-1, XIPiT-1, and hPiT-2 by using 0.3 mM cold $P$ with or without 1 mM arsenate. Arsenate induced a significant reduction in $P$ transport in the two PiT-1 isoforms. However, arsenate did not affect $P$ transport by hPiT-2 (Fig. 8).

The reduction in $^{32}P$ uptake in the presence of arsenate may have resulted from inhibition or substitution of arsenate for $P$. To investigate these possibilities further, we studied the transporter behavior by electrophysiology. Under voltage clamp, arsenate induced currents in oocytes expressing XIPiT-1. Applying 1 mM arsenate elicited an inward current that was $\sim 40\%$ of current induced by 1 mM $P$ (Fig. 8B). This suggested that arsenate is indeed transported by XIPiT-1. In contrast, no significant change in the holding current was observed when applying sulfate (1 mM; Fig. 8B). We then measured the apparent arsenate affinity constant ($K_{arsenate}$) in XIPiT-1-expressing oocytes and normalized the current response to that obtained with 1 mM $P$, for each oocyte. Fitting the data in Fig. 8C with Eq. 1 gave a $K_{arsenate}$ of 0.83 ± 0.10 mM, which indicated that the arsenate affinity of XIPiT-1 was significantly smaller than its affinity for $P$ (0.19 ± 0.02 mM at pH 7.4 in Fig. 7A). In addition, the maximum attainable arsenate current ($I_{arsenate}^{\max}$), as reported by the fit of Eq. 1 to the arsenate data, was smaller compared with $P$ (0.74 compared with 1.2 for similarly normalized data), which indicated that the transporter transports arsenate at a slower rate than $P$.

Lithium is known to support transport in some $Na^+$-coupled transporters such as the $Na^+$-dicarboxylate transporter (42) and $Na^+$-driven Cl$^-$/HCO$^-$ exchanger (54). Our results from the $^{32}P$ uptake experiment (Fig. 1A) and others (6, 53) indicated that $P$ transport through PiT isoforms could be driven by $Li^+$. We revisited this question by using electrophysiology in oocytes expressing XIPiT-1. We recorded currents induced by 1 mM $P$ in 100 mM Na$^+$, 100 mM Li$^+$, and 100 mM choline$^+$ solutions at $V_h = -50$ mV. Under these conditions, $P$ elicited a current response in Li$^+$ that was $\sim 15 \pm 1\%$ of the response seen in Na$^+$, whereas in 100 mM choline, $P$ did not elicit inward currents (data not shown). Thus it appears that Li$^+$ also can act as a substrate for PiT, whereas choline is excluded.

**Inhibitors of PiT**

PFA is a well-documented competitive inhibitor of the type II $Na^+P$ cotransporter (7, 47) and has also been reported to inhibit PiT-mediated $P$ uptake (3, 49). Recently, Villa-Bellosta et al. (53) showed by using $^{32}P$ uptake assays that PFA is a very poor inhibitor of $P$ uptake mediated by rat PiT-1 and PiT-2 both in oocytes and in native rat vascular smooth muscle cells. We investigated the effect of PFA at a concentration of 1 mM on oocytes expressing PiT proteins by means of $^{32}P$ uptake and electrophysiology. First, we measured $^{32}P$ uptake in uninjectected oocytes and oocytes expressing hPiT-1, XIPiT-1, or hPiT-2 with 0.3 mM cold $P$ with or without 1 mM PFA. Figure 9A shows that PFA did not cause any statistically significant differences in $P$ uptake in any of the isoforms studied.

Similarly, under voltage clamp at $-50$ mV, XIPiT-1-expressing oocytes showed no difference in electrogenic activity when 1 mM PFA was applied in the presence of 0.3, 0.1, or 0.03 mM PFA, compared with the response to $P$ alone (Fig. 9, B and C). PFA induced a small upward deflection of the holding current that we also observed for uninjected oocytes. We also

![Fig. 8. Arsenate and sulfate (SO$_4^{2-}$) as potential PiT substrates. A: $^{32}P$ uptake was performed in control oocytes (NI) and in oocytes expressing hPiT-1, XIPiT-1, or hPiT-2 by using ND100 solution with 0.3 mM P, with or without 1 mM arsenate. Arsenate significantly decreased P uptake for both PiT-1 clones but not for hPiT-2. B: current response to 1 mM $P$, 1 mM arsenate, and 1 mM SO$_4^{2-}$ was measured in oocytes expressing XIPiT-1 at $V_h = -50$ mV. Data from each oocyte were normalized to current response in 1 mM $P$. C: current response to different concentrations of arsenate was measured in oocytes expressing XIPiT-1 at $V_h = -50$ mV. Arsenate-induced current was normalized to value obtained with 1 mM $P$, for each oocyte and was plotted as a function of arsenate concentration. Solid line indicates fit with Eq. 1.](http://ajpcell.physiology.org/ by 10.220.33.5 on April 1, 2017)
observed no effect of PFA on currents elicited by 0.3 mM Pi in voltage-jump experiments, which extended the voltage range examined (Fig. 9D). As a positive control, an oocyte from the same donor frog that expressed the flounder NaPi-IIb gave 75% inhibition at −100 mV (data not shown). Thus we can conclude that, in contrast to its action as a competitive inhibitor of type II Na\(^+\)-Pi cotransporters, PFA does not inhibit Pi transport mediated by PiT.

Technetium-99m dimercaptosuccinic acid [\(^{99m}\)Tc-(V)-DMSA] is a radiopharmaceutical agent with potential in the medical imaging of tumors. Recently, Denoyer et al. (11) reported that entry of \(^{99m}\)Tc-(V)-DMSA in tumor cell lines is mediated by PiT transporters. We investigated whether DMSA could affect \(I_\Pi\) or whether we could observe DMSA-induced currents in XlPiT-1-expressing oocytes. We observed no effect of 1 mM DMSA alone nor any effect of 1 mM DMSA on currents induced by 0.1 mM Pi in XlPiT-1-expressing oocytes at either pH 6.2 or 7.4 (data not shown), indicating that DMSA is neither transported by nor able to block Pi transport mediated by XlPiT-1. It is, however, possible that an interaction of DMSA with PiT transporters requires chelation with technetium [Denoyer et al. (11)] only used \(^{99m}\)Tc-(V)-DMSA, not DMSA alone, in their studies]. This question will remain open for further studies.

**Pre-Steady-State Kinetics**

Pre-steady-state current relaxations induced by voltage steps are a common property of electrogenic members of the SLC34 Na\(^+\)-Pi cotransporter family (NaPi-IIa/b; reviewed in Ref. 21), and relaxations in the millisecond range are readily observed superimposed on the oocyte capacitive-charging transient. We applied the same experimental protocols to XlPiT-1-expressing oocytes (e.g., Refs. 17 and 55); however, from the raw data alone for oocytes exhibiting comparable \(I_\Pi\) (−200 nA) as observed for the type II cotransporters, we were unable to distinguish pre-steady-state relaxations from the endogenous response either in ND100 or ND0 (not shown).

At saturating concentrations, Pi is known to suppress pre-steady-state charge movements (17), and therefore, in a further attempt to detect charge movements, we subtracted records in ND100 from the corresponding traces in ND100 + 1 mM Pi, and then examined the time course of currents at the voltage-step onset at high time resolution (Fig. 10A). The current did not change immediately at the onset of the voltage step, and we observed a finite time dependence as the current settled to the new steady-state value. This was qualitatively similar to the time course documented for the type II Na\(^+\)-Pi cotransporter (e.g., Ref. 24) under similar experimental conditions. Moreover, the relaxations extended beyond the range of the membrane-charging time (1–2 ms) and were therefore unlikely to have originated from a voltage-clamp artifact. For uninjected oocytes from the same donor frog, no relaxations were observed under the same experimental conditions (data not shown). Interestingly, the apparent charge movements were not balanced, i.e., the area under the relaxation curve was not the same for the ON and OFF steps as illustrated in Fig. 10A for two traces at extreme hyper- and hypopolarizing potentials. Under the assumption that we had detected all transient charges for both steps, the lack of charge balance suggested that the relaxations do not arise from a conserved charge movement within the transmembrane field. Nevertheless, the apparent charge associated with the ON transition correlated with \(I_\Pi\) over a wide range of expression levels (\(n = 12\)), which strongly suggested that the relaxations were associated with the presence of XlPiT-1 in the membrane (Fig. 10C).
DISCUSSION

We have performed a thorough characterization of the transport kinetics of PiT. Previous studies used mammalian PiT-1 and PiT-2 isoforms expressed in Xenopus oocytes or various cell lines and assayed transport activity by radionuclide uptake. The apparent $K_V^{0.5}$ reported in these studies lay between 50 and 500 $\mu$M for measurements in cell lines (14, 33, 41, 48, 49, 60) and between 40 and 300 $\mu$M in oocytes (3, 4, 48). Whereas cell-specific environmental issues might influence $K_V^{0.5}$ measured in different environments, it is also most likely that the lack of membrane-potential control as well as contamination from endogenous $P_i$-transport systems may have contributed to the spread in the measurements. The problem is further exacerbated by the low $P_i$-transport levels attained in these systems, possibly explaining why only limited electrophysiological characterization has previously been carried out (32, 33).

Reasoning that Xenopus oocytes might express a Xenopus protein better than a mammalian one, we compared the $P_i$ transport levels of XIPIT-1, hPiT-1, and hPiT-2 by heterologous expression in Xenopus oocytes. Indeed, our results show that XIPIT-1 expressed far better than either of the mammalian isoforms, and therefore we performed most of our kinetic characterization on XIPIT-1. At the amino acid level, XIPIT-1 is ~78% identical to hPiT-1 and ~60% identical to hPiT-2, and it is possible that the isoforms differ to some extent in their kinetic profiles. However, the main transport-function characteristics, such as substrate specificity, binding order, transport stoichiometry, pH sensitivity, and voltage dependence are probably very similar in the different isoforms, given the high degree of amino acid identity, thus validating the use of XIPIT-1 as a model for vertebrate PiT-1 characteristics in general.

Transport Stoichiometry Determination

Our results from the simultaneous $^{22}\text{Na}$ vs. $^{32}\text{P}$ and charge vs. $^{32}\text{P}$ uptake experiments strongly suggest that the transport cycle of XIPIT-1 involves the transport of 2 $\text{Na}^+$ ions, one $P_i$ in the form of the monovalent $\text{H}_2\text{PO}_4^-$ ion, and one net positive charge. Because the transport stoichiometries were the same, whether measured at pH 6.2 ($\text{H}_2\text{PO}_4^-:\text{HPO}_4^{2-}$ ratio 4:1) or at pH 7.4 ($\text{H}_2\text{PO}_4^-:\text{HPO}_4^{2-}$ ratio 1:4), it appears that the transporter has an absolute preference for monovalent $P_i$. Furthermore, $\text{pH}$ changes recorded at the oocyte surface are consistent with transport of $\text{H}_2\text{PO}_4^-$. This contrasts with the behavior of the flounder NaPi-IIb, for which surface $\text{pH}$ changes were consistent with transport of $\text{HPO}_4^{2-}$. Thus the results from the surface $\text{pH}$ measurements are in excellent agreement with the stoichiometry measurements from simultaneous radionuclide uptake and voltage-clamp experiments both for type II (22, 55) and type III (this study) $\text{Na}^+:\text{P}$ cotransporters.

A 2:1 $\text{Na}:\text{P}$ transport stoichiometry for PiT was initially proposed by Kavanaugh and Kabat (32), but the experimental data were never published. Using $^{32}\text{P}$ uptake experiments in oocytes expressing mouse PiT-2, Bai et al. (3) observed that the $\text{Na}^+$ dependence of $P_i$ transport showed a very weak positive cooperativity with a $n_H$ of 1.1. This low cooperativity agrees with our electrophysiological findings for XIPIT-1, in which the cooperativity of $\text{Na}^+$ binding became apparent only at pH 8.0, where the $\text{H}_2\text{PO}_4^-$ concentration is 0.059 mM. At higher $\text{H}_2\text{PO}_4^-$ concentrations, we observed no cooperativity for the $\text{Na}^+$ dependence (see Fig. 3), and fitting the data with a Michaelian function was statistically indistinguishable from a free fit to the Hill equation. Recently, Saliba et al. (43) showed elegantly that the intraerythrocytic malaria parasite Plasmodium falciparum uses a type III $\text{Na}^+:\text{P}$ cotransporter (PfPiT) to mediate $\text{Na}^+$-driven $P_i$ uptake. By using isolated parasites, they estimated a $n_H$ of 2.1 for the $\text{Na}^+$ dependence of $^{32}\text{P}$ influx, and by using a “static head” experiment, they confirmed the 2:1 $\text{Na}^+:\text{P}$ stoichiometry.

Thus the 2:1 $\text{Na}^+:\text{H}_2\text{PO}_4^-$ stoichiometry of PiT has been unequivocally demonstrated in these studies. Because PiT proteins transport $\text{H}_2\text{PO}_4^-$, we would expect that this would cause a concomitant acid loading of the cell. In contrast, type II $\text{Na}^+:\text{P}$ cotransporters, which mediate $\text{HPO}_4^{2-}$ transport, would alkalinize the cell. Whether the transport rates of type II or type III $\text{Na}^+:\text{P}$ cotransporters in their natural environments are high enough to affect cell $\text{pH}$ is not known and has not, to our knowledge, been studied in any cell system. Our own
experience with expressing type II and type III Na\(^+\)-P\(_i\) cotransporters in Xenopus oocytes would suggest that the pH changes would be minimal at most. However, with respect to acid-base regulation in general, the question as to which P\(_i\) species is preferential might be of some consequence. For example, in the average human body, \(~70\%\) of filtered P\(_i\) is reabsorbed in the form of divalent HPO\(_4\)\(^2-\) in the kidney proximal tubule via type II Na\(^+\)-P\(_i\) cotransporters. Because H\(_2\)PO\(_4\) remains, this effectively constitutes a “secretion” of H\(^+\) into the primary urine. For a glomerular filtration rate of 180 l/day, a serum P\(_i\) concentration of 1 mM, and a pH of 7.4 in the lumen of the proximal tubule, we estimate that \(~40\) mmol of H\(^+\) a day is “excreted” into the proximal tubular lumen this way.

Other Substrates

Anions. In addition to Na\(^+\) and H\(_2\)PO\(_4\)\(^-\), it appears that PiT-1 and PiT-2 transport few other substrates, with the exception of arsenate. Arsenate is a phosphate mimetic and is highly toxic to cells because it can substitute for P\(_i\) in glycolytic and cellular respiration pathways. Accordingly, it is transported both by type II Na\(^+\)-P\(_i\) cotransporters (7, 27) and PiT (this study). In both transporter families, K\(_{\text{arsenate}}\) is three to four times higher than K\(_{\text{oPi}}\) and the transport rate of arsenate is slower than that for P\(_i\), showing that it is not a perfect P\(_i\) substitute. In contrast to arsenate, SO\(_4\) does not induce any currents in XIPiT-1-expressing oocytes.

Cations. In PiT proteins, Li\(^+\) ions can act as cosubstrates, as evidenced by both tracer flux (6, 53) and electrophysiology, but the transport rate is much lower than for Na\(^+\) (the magnitude of the I\(_p\) in XIPiT-1 using 1 mM P\(_i\) in 100 mM Li\(^+\) solution was only \(~15\%\) of that in 100 mM Na\(^+\)). We do not know if this is because the affinity for Li\(^+\) is much lower than that for Na\(^+\) or because Li\(^+\) is transported less efficiently than Na\(^+\), because the currents were too small to carry out a dose-dependence measurement. Other cations, such as choline or K\(^+\) (53), do not support P\(_i\) transport.

Protons. Protons serve as the driving ion in PiT family members from plants and bacteria (5), whereas in vertebrates dependence measurement. Other cations, such as choline or K\(^+\) (53), do not support P\(_i\) transport.

PFA does not inhibit PiT

PFA is a well-known competitive inhibitor of Na\(^+\)-dependent P\(_i\) transport (35, 47). Inhibition studies using heterologously expressed type II Na\(^+\)-P\(_i\) cotransporters have unequivocally shown that PFA inhibits NaPi-II-mediated P\(_i\) transport (7, 61). In contrast, only limited data is available on the effect of PFA on PiT proteins. Bai et al. (3) expressed mouse PiT-2 in Xenopus oocytes and documented a 40% decrease in 32P\(_i\) uptake by using a 50-fold excess of PFA (5 mM). Recently, Villa-Bellosa et al. (53) reported that high concentrations of PFA (\(\geq2.5\) mM, \(\geq50\)-fold excess of PFA) reduced P\(_i\) uptake in smooth muscle cells and in oocytes expressing PiT-1 or PiT-2.

We found no effect of PFA on 32P\(_i\) uptake in any of the PiT isoforms studied and no effect of PFA on I\(_p\) mediated by XIPiT-1 (Fig. 9C), which confirms the findings of Villa-Bellosa et al. (53) with 1 mM PFA found by uptake assays alone. To avoid nonspecific effects, we did not use concentrations of PFA > 1 mM, because these usually increase the oocyte endogenous leak (I. C. Forster, unpublished experiments). Recently, it was reported that PFA inhibits P\(_i\)-induced calcification in smooth muscle cells (31, 34) and that it inhibits matrix calcification in osteoblast-like cells (46). Although other experiments carried out by Li et al. (34) and Suzuki et al. (46) point to an important role of PiT in the calcification process, the effect of PFA in inhibiting calcification is probably more related to the ability of phosphonates and bisphosphonates to inhibit the formation of calcium phosphate crystals (15, 16, 59) than to inhibition of P\(_i\) transport through PiT. Alternatively, perhaps PFA-inhibitable type II Na\(^+\)-P\(_i\)
cotransporters play a more important role in mineral formation than previously thought (37).

PiT-1 Substrate-Binding Scheme

Our substrate-dependence data strongly suggest that XIPI-T-1 binds substrates in an ordered manner, as indicated by the dependence of $K_{0.5}$ on Na$^+$ concentration (Fig. 5F) and $K_{0.5}$ on Pi$_i$ (Fig. 4F) (45). For Pi$_i$ as the variable substrate, the dependence of $P_{\text{max}}$ on Na$^+$ is consistent with Na$^+$ being the last substrate to bind. Consistent with the 2:1 Na$^+$/Pi stoichiometry, a possible binding order, based on analogy with the electrogenic SLC34 transporters, might therefore be Na$^+$/H$_2$PO$_4^-$:Na$^-$. The increase in apparent cooperativity for Na$^+$ interaction observed at pH 8.0 would also support this scheme, whereby the decreased availability of H$_2$PO$_4^-$ at pH 8.0 would increase the apparent dissociation constant associated with the proposed first Na$^+$-binding step, thus conferring a greater cooperativity to the overall Na$^+$ interaction. On the other hand, the $V_{\text{max}}$ effect observed for Pi$_i$ with Na$^+$ as the variable substrate (Fig. 4F), would not be consistent with this scheme and suggests that a more complex binding/debinding mechanism exists that may involve both ordered and random partial reactions.

The voltage dependence of PiT requires that at least one partial reaction in the transport cycle is $V_m$ dependent. This implies that mobile charges must sense the $V_m$, for example charged amino acid residues intrinsic to the protein or charged substrates (e.g., Na$^+$) moving within the transmembrane electric field. For example, the decrease of $K_{0.5}$ with hyperpolarizing potentials (Fig. 4F) suggests that Na$^+$ interaction with the transporter is voltage dependent, whereby a more negative $V_m$ would increase the likelihood of Na$^+$ binding. Alternatively, Saliba et al. (43) have proposed a model for PiPIT whereby the empty carrier translocation from inward- to outward-facing conformations involves movement of an intrinsic negative charge; however, they provided no direct experimental evidence in support of this proposal. Indeed, for many cation-driven cotransport systems, pre-steady-state measurements in the absence of one or both substrates have revealed transporter-associated charge movements, which provide convincing evidence of voltage-dependent partial reactions in the transport cycle (13, 18, 36, 38, 44, 55, 58). For XIPI-T-1, our inability to resolve pre-steady-state relaxations in oocytes expressing XIPI-T-1 means that we cannot assign voltage dependence to either the empty carrier or first Na$^+$-binding partial reactions in the transport cycle. However, we cannot exclude the possibility that pre-steady-state currents in XIPI-T-1-expressing oocytes were too small or too fast to be detected and so remain buried in the oocyte capacitive-charging transient. Typically, type II Na$^+$/Pi$_i$ cotransporters that exhibit similar steady-state transport activity show ~10-fold larger charge movements (e.g., Ref. 21). Based on our findings, we therefore propose a kinetic scheme for PiT in which the reorientation of the empty carrier and the interaction of the first Na$^+$ ion are electrically silent, and we tentatively conclude that voltage dependence arises from either the second Na$^+$ interaction, the final translocation of the fully loaded carrier, or both.

Conclusions

The novel electrogenic kinetics of the members of the PiT family present a contrasting view of Na$^+$-driven Pi$^{-}$cotransport with respect to that of the well-characterized members of the SLC34 family (NaPi-IIa/b), and these kinetic differences are underscored by the lack of sequence homology at the molecular level. First, the 2:1 vs. 3:1 Na$^+$/Pi stoichiometry indicates a 10-fold weaker concentrating ability of SLC20 compared with electrogenic SLC34 family members. Second, the preference of PiT for monovalent over divalent Pi implies that Pi transport by the two transporter families have opposite effects on intra- and extracellular pH. Third, the relative insensitivity to pH compared with members of the SLC34 family suggests that the PiT are more tolerant of the physiological conditions, and this may also reflect their ubiquitous housekeeping role. Finally, the finding that PFA does not inhibit PiT underscores the need for a more efficacious blocker that, ultimately, could be used in the treatment of patients with hyperphosphatemia-induced vascular calcification.

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