The leak mode of type II Na\(^+-\)P\(_i\) cotransporters

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**Abbreviations:** \(I_p\), P\(_i\)-dependent change in holding current; \(I_{PFA}\), PFA-dependent change in holding current; MTS, methanethiosulfonate; MTSEA, 2-aminoethyl MTS hydrobromide; MTSES, (MTS ethylsulfonate); MTSET, 2-(triethylammonium)ethyl MTS bromide; NaPi-IIa/b/c, type IIa/b/c sodium phosphate cotransporter; PFA, phosphonoformic acid; P\(_i\), inorganic phosphate; SCAM, substituted cysteine accessibility method; TMD, transmembrane domain; WT, wild-type; SLC34, solute carrier family 34

**Key words:** phosphate, sodium, cotransport, leak current, Xenopus oocyte, electrophysiology

Na\(^+-\)coupled phosphate cotransporters of the SLC34 gene family catalyze the movement of inorganic phosphate (P\(_i\)) across epithelia by using the free energy of the downhill electrochemical Na\(^+\) gradient across the luminal membrane. Electrogenic (NaPi-IIa/b) and electroneutral (NaPi-IIc) isoforms prefer divalent P\(_i\) and show strict Na\(^+\)-P\(_i\) stoichiometries of 3:1 and 2:1, respectively. For electrogenic cotransport, one charge is translocated per transport cycle. When NaPi-IIa or NaPi-IIb are expressed in Xenopus oocytes, application of the P\(_i\) transport inhibitor phosphonoformic acid (PFA) blocks a leak current that is not detectable in the electroneutral isoform. In this review, we present the experimental evidence that this transport-independent leak originates from a Na\(^+\)-dependent unipporter mode intrinsic to NaPi-IIa/b isoforms. Our findings, based on the characteristics of the PFA-inhibitable leak measured from wild type and mutant constructs, can be incorporated into an alternating access class model in which the leak and cotransport modes are mutually exclusive and share common kinetic partial reactions.

**Introduction**

Secondary-active cotransporters catalyze uphill solute movement across membranes by using the free energy available from the electrochemical gradient of the driving substrate, usually a monovalent cation (Na\(^+\), H\(^+\) or K\(^+\)). The transport cycles of many members of this class are electrogenic, whereby net charge transfer accompanies cotransport and the partial reactions that constitute the transport cycle show voltage dependent kinetics. We can investigate the kinetics of these carriers by electrophysiological techniques, as ideally the substrate-induced current is an indirect measure of the transport rate for a constant number of charges translocated per cycle. Uncoupled currents give rise to deviations from the tightly coupled electrogenicity and strict stoichiometry between driving and driven substrates that is expected of the ideal secondary-active carrier. They are also referred to as leak or slippage currents (reviewed in refs. 1 and 2) and are intimately associated with the heterologous expression of the carrier protein. Their coexistence alongside the coupled transport electrogenic activity has led to a reassessment of the traditional view of channels and carriers as unique molecular entities (reviewed in ref. 3).

Uncoupled currents are commonly sub-classified as transport-dependent and transport-independent currents, depending on whether they are detectable in the absence or presence of substrate. Cotransporters can exhibit one or both types of uncoupled current with different contributing ions. Uncoupled currents appear to be ubiquitous among electrogenic carriers and they have been described for gene products of at least nine solute carrier families identified in the human genome (Table 1). Within a given solute carrier family, the properties of the uncoupled currents can also vary considerably for different isoforms, measured under the same experimental conditions. For example, in the biogenic amine transporter subgroup of the SLC6 family, GAT4 is reported to have two transport independent, mutually exclusive Na\(^+\)-dependent conductances that are permeable to Li\(^+\) or Cl\(^-\), both of which are present only in the absence of external Na\(^+\) and GABA. In contrast, the human GAT1 isoform displays only a Li\(^+\)-dependent uncoupled leak [Uncoupled currents are not exclusively associated with electrogenic transport. Electroneutral transporters show uncoupled currents, for example, Na\(^+\)-coupled glutamine transporter (SNAT3)].

Transport-dependent uncoupled currents are observed in the presence of both driven and driving substrates and manifest themselves as currents in excess of those associated with the stoichiometric charge translocation. The most notable examples are members of the excitatory amino acid transporter family (EAAT’s of the SLC1 family) that catalyze transport of glutamate driven by two Na\(^+\) ions. For these carriers, experimental manipulations allow separation of the transport-dependent current to be separated into a stoichiometric component and a thermodynamically uncoupled anion selective component that is only activated in the presence of Na\(^+\) and the amino acid (e.g., glutamate, aspartate). This uncoupled current has been likened to that of ligand-gated channel that is gated by the driven substrate. Although the presence of Cl\(^-\) ions is not essential for cotransport, it is accelerated by Cl\(^-\) ions and studies suggest...
that the negative charge acts as a counterbalance to the excess charge associated with cotransported Na⁺ ions.¹⁴

In contrast, transport-independent uncoupled currents are observed in the absence of the driven substrate and represent a constitutive leak that may or may not involve the driving substrate. In some cases, for example the Na⁺/I⁻ cotransporter (NIS)¹⁵ and the Na⁺/L-ascorbic acid transporters (SVCT1,2),⁶ the leak currents measured when the transporter is heterologously expressed in Xenopus oocytes are significantly larger than those observed for control oocytes and comparable in magnitude to the substrate-induced current. For other cotransporters, the currents can only be quantified with confidence when the transporter is heterologously expressed in Xenopus oocytes, as well as functionally reconstituted carriers, together with 3-D crystallographic data from bacterial homologs, is helping to provide clearer answers to these questions.

Indirect support that uncoupled currents are intrinsic to carriers comes from the observation that their magnitude correlates strongly with that of the substrate-induced electrogenic response, which should reflect the number of active transporters expressed in the membrane. However, it has been reported that heterologous expression of proteins in Xenopus oocytes can lead to the upregulation of endogenous Cl⁻ and non-specific hyperpolarization-activated cation channels (reviewed in refs. 22–25). Therefore, the number of upregulated endogenous channels might still correlate with the activity of the heterologously expressed protein. More compelling evidence that leak currents are intrinsic to the carrier protein comes from studies in which the leak is modified or even “uncoupled” from the cotransport mode activity through mutations of the cotransported substrates, either the same permeation pathway as the cotransported substrates, or a pore established by oligomerization of functional subunits. Evidence from structure-function studies on heterologously expressed carriers, together with 3-D crystallographic data from bacterial homologs, is helping to provide clearer answers to these questions.

Several questions arise with respect to the identity and underlying mechanism of uncoupled currents:

(i) Are they an intrinsic property of the carrier or an artifact of the heterologous expression system?

(ii) Given that they are constitutive to the carrier, do they share either the same permeation pathway as the cotransported substrates, or are the ions conducted via a separate pathway, for example through a pore established by oligomerization of functional subunits?

(iii) Is the leak better described as a channel (gated pore) or a uniport (carrier) type mechanism?

(iv) Do they play mechanistic or physiological roles?

It has been challenging to answer these questions unambiguously, in part because of possible artifacts of the expression systems, the small size of the currents compared with the cotransport mode currents and given that the same ions are cotransported and permeate the leak pathway. Evidence from structure-function studies on heterologously expressed, as well as functionally reconstituted carriers, together with 3-D crystallographic data from bacterial homologs, is helping to provide clearer answers to these questions.

### Table 1 Examples of gene products of the solute carrier families that mediate uncoupled currents

| SLC | Example proteins | Coupled substrates | Un-coupled current | Ions carried by uncoupled currents | Selected references |
|-----|------------------|--------------------|--------------------|-----------------------------------|---------------------|
| 1   | EAAT1,2,3,4      | Na⁺/H⁺/K⁺/Glu      | T                  | Cl⁻                              | (11, 69)            |
|     | EAAC1            | L                  | Na⁺                | (70)                             |
|     | ASC1             | T                  | K⁺, H⁺             | (8, 9)                           |
| 5   | SGLT1            | Na⁺/glucose        | L                  | Na⁺                             | (17, 67, 71)        |
|     | NIS              | Na⁺/I⁻            | L                  | Na⁺                             | (15)                |
|     | SMIT             | Na⁺/myoinositol    | L                  | Na⁺                             | (72)                |
|     | SMCT             | Na⁺/monocarboxylate| L                  | Anions                          | (73)                |
|     | CHT              | Na⁺/choline        | L                  | Na⁺                             | (74)                |
| 6   | GAT1,4           | Na⁺/Cl⁻/GABA       | L                  | Li⁺,Cs⁺, Cl⁻                     | (5, 19, 75)         |
|     | SERT             | Na⁺/Cl⁻/K⁺/SHMT    | T/L                | Na⁺, Li⁺, K⁺, H⁺                 | (7, 75, 76)         |
|     | hDAT             | Na⁺/Cl⁻/DA⁺        | T/L                | Na⁺, Li⁺, K⁺, H⁺, Cl⁻            | (20, 77)            |
| 11  | DMT1             | H⁺/divalent cations| L                  | H⁺                              | (34, 49)            |
| 13  | NaD (NaC)        | Na⁺/dicarboxylates  | L                  | Na⁺                             | (78)                |
| 15  | PepT2            | H⁺/peptides        | L                  | H⁺                              | (79)                |
| 23  | SVCT1,2          | Na⁺/ascorbic acid  | L                  | Na⁺                             | (16, 80)            |
| 34  | NaPi2a/b         | Na⁺/Pₗ            | L                  | Na⁺                             | (41, 45)            |
| 38  | SNAT3            | Na⁺/glutamine      | T                  | K⁺, H⁺, Na⁺/H⁺ exh.             | (6)                 |

¹Nomenclature according to Human Genome Organisation Database (4). ²According to the nomenclature of Sanders and Amara (2): L(eak) = transport-independent currents, T(ransport) = transport-dependent currents respectively. ³Only studies using the Xenopus oocyte expression system are cited.
As some cotransporters are known to form homo-oligomers, there has been speculation that leak pathways could be created at the interface between the subunits analogous to the structure of multimeric ionotropic proteins (reviewed in ref. 35). However, recent studies on the neuronal EAAT3,36 and its bacterial homolog glutamate transporter GLTPH12 suggest that the translocation pathways for the substrate itself and transport-dependent leak are colocalized to individual subunits and function independently. On the other hand, direct structural evidence concerning the localization of the permeation pathway for transport-independent uncoupled currents is lacking.

The Uncoupled Leak of SLC34 Proteins

Introduction. Members of the SLC34 family of Na+-coupled inorganic phosphate (Pi) transporters play a vital physiological role in Pi homeostasis, particularly in the kidney, where they are responsible for Pi reabsorption from the glomerular filtrate across the apical membrane of proximal tubule epithelia. They are also expressed in epithelial membranes in the small intestine and other organs (testes, lung, liver), where their physiological function is less well understood (reviewed see ref. 37). The family comprises three isoforms that prefer divergent Pi, as the driven substrate: NaPi-IIa, NaPi-IIb catalyze electrogenic transport with a Na+:Pi stoichiometry of 2:1 and one net charge is translocated per transport cycle;38,39 NaPi-IIc is electroneutral and therefore cotransports with a Na+:Pi stoichiometry of 2:1.40

Based on kinetic studies of WT isoforms and structure-function studies of mutant constructs, we have developed a kinetic scheme for the cotransport cycle (Fig. 1A) that comprises a sequence of partial reactions representing the transitions between unique conformational states of the protein.37,41-43 Under normal physiological conditions, the cotransport cycle begins in the outward facing conformation of the empty carrier (state 1), which favors ordered binding of two Na+ ions, followed by binding of one divalent Pi and a 3rd Na+ ion from the external medium. A translocation partial reaction of the fully loaded carrier (transition 2a) is sufficient for electrogenic transport with a Na+:Pi stoichiometry of 3:1 and one net charge per cycle (Fig. 1A). In this model, both the cotransport and leak modes mediate the net movement of one charge per cycle (Fig. 1A, inset). For the cotransport mode, this has been established by simultaneous isotope flux and electrophysiological recording.38,39 For NaPi-IIa/b isoforms, it has not been feasible to apply the same approach to establish the stoichiometry of the leak because the corresponding cation flux is at the limit of experimental resolution using isotopes. Therefore, our current understanding of the leak mechanism and the evidence to support of its inclusion in the kinetic scheme as a unipporter has come from the integration of findings from electrophysiological studies using both WT and mutant constructs as described below.

Properties of the uncoupled leak for WT NaPi-IIa/b. We have found no evidence of significant electrogenic activity in excess of the stoichiometrically coupled components for three electrogenic isoforms of the SLC34 family38,39 when expressed in Xenopus oocytes. However, a transport-independent leak current was postulated by comparing the electrogenic responses of oocytes heterologously expressing NaPi-IIa or NaPi-IIb isoforms to Pi, or the Pi-transport inhibitor phosphenforderformic acid (PFA). Figure 1B depicts a representative recording from an oocyte expressing rat NaPi-IIa when voltage was clamped to -50 mV using a two-electrode voltage clamp (inset). In contrast to the electrogenic response to a saturating concentration of Pi (1 mM), which results in a downward deflection in the holding current corresponding to the inward movement of charge (Ipi, Fig. 1B), 3 mM PFA induces a small upward deflection of the steady-state holding current (I_{PFA}). This is interpreted as evidence of block of an intrinsic leak, akin to the phlorizin-dependent block of the leak intrinsic to the Na+-coupled glucose transporter (SGLT1), first described by Umbach et al.57 The holding current level reached during PFA superfusion is not zero, but is given by the endogenous leak current of the oocyte (I_{Ende}), which at a membrane potential of -50 mV, is usually inward but its magnitude can vary widely both between and within batches of oocytes. In the absence of external Na+, PFA like Pi, does not induce a change in holding current, which underscores the Na+-dependence for the electrogenic responses to Pi and PFA. At -50 mV, I_{PFA} accounts for approx 10–20% of I_{P_i}, although we have documented larger PFA-induced changes in holding current for constructs that contain point mutations at sites associated with the putative transport pathway (see below). We have also observed a PFA-dependent block of holding current in all characterized electrogenic NaPi-II isoforms, however at a given membrane potential its magnitude relative to I_{P_i} can vary, which is also reflected in differences in steady-state voltage dependence of the cotransport mode (see Fig. 5C).

As we first reported,41 the magnitude of I_{PFA} correlates with I_{P_i} under the same voltage clamp conditions (Fig. 1C). For the rat NaPi-IIa isoform I_{P_i} is typically < -200 nA at -100 mV, whereas the magnitude of the leak current is comparable to I_{Ende} and therefore could not be easily detectable without using PFA as a blocking substrate. As illustrated for a representative oocyte expressing rat NaPi-IIa (lower, Fig. 1D), the current-voltage (I-V) relation for

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Leak in phosphate transporters
Leak in phosphate cotransporters

Figure 1. The transport modes of electrogenic SLC34 proteins. (A) Kinetic scheme showing the ordered sequence of partial reactions that result in net charge translocation for the cotransport mode (green shading) and leak mode (violet shading). Two electrogenic partial reactions (blue) account for the voltage dependence of transport. All other partial reactions are assumed to be electroneutral. Inset shows the two modes of electrogenic transport: cotransport and leak. Both involve the translocation of one net charge per cycle. Only the stoichiometry of the cotransport mode has been determined experimentally by simultaneous uptake and voltage clamp.38 (B) Electrogenic responses of an oocyte expressing the rat NaPi-IIa isoform to saturating Pi (1 mM) \( (I_{\text{Pi}}) \) and PFA (3 mM) \( (I_{\text{PFA}}) \) in the presence of 100 mM Na+. Oocyte is voltage clamped using a two-electrode voltage (inset) and the membrane clamped to -50 mV. \( I_{\text{Endog}} \) represents the endogenous oocyte current. If the leak is fully suppressed by saturating Pi, the predicted electrogenic cotransport activity is given by \( I_{\text{Cot}} \) (Forster IC, unpublished data). (C) Correlation of \( I_{\text{PFA}} \) with \( I_{\text{Pi}} \) shown for oocytes harvested from >5 donor frogs at two holding potentials (-100 mV, filled squares; -60 mV, open squares). Linear regression lines have slopes \(-0.16 \pm 0.03 \) (-100 mV); \(-0.11 \pm 0.02 \) (-60 mV) but were not statistically significant \( (n = 29) \). (D) Typical steady-state voltage dependence of \( I_{\text{Pi}} \) and \( I_{\text{PFA}} \) for a representative oocyte expressing rat NaPi-IIa (lower) and a non-injected (NI) oocyte from the same donor frog as control (upper). Each data point is obtained by subtracting the oocyte holding current in the control solution from the current with 1 mM Pi or 1 mM PFA added to the control solution. Note that \( I_{\text{PFA}} \) reverses at \(-10 \text{ mV} \) for the NaPi-IIa expressing oocyte (arrow). Dotted line indicates the apparent PFA-inhibitable leak current \(-I_{\text{PFA}} \), see text). An endogenous response to PFA is also observed for NI for \( V > 0 \text{ mV} \) (filled squares: \( I_{\text{Pi}} \); open squares: \( I_{\text{PFA}} \)). (Andrini O and Forster IC, unpublished data).

\( I_{\text{Pi}} \) shows an inward current for all test potentials, consistent with cotransport cycling clockwise in Figure 1A. In contrast, \( I_{\text{PFA}} \) is outward for hyperpolarizing potentials and typically reverses at potentials between -30 mV to -10 mV (arrowed, Fig. 1D). This apparently anomalous \( I-V \) behavior for a substrate-dependent current is simply a result of subtracting the holding current at each potential in the absence of PFA from the response in the presence of PFA to eliminate \( I_{\text{Endog}} \) [We adopt the convention that \( I_{\text{P}} \) and \( I_{\text{PFA}} \) indicate the change in holding current caused by the addition of \( \text{P}_i \) or PFA to the control superfusate. For steady-state \( I-V \) curves, data points were obtained by subtracting currents recorded in response to voltage steps in the presence of substrate, from the corresponding records in the control solution, thereby eliminating endogenous currents.

The validity of this method rests on the assumption that substrates do not modulate endogenous currents]. The apparent leak current, which we assume PFA fully inhibits, is therefore \(-I_{\text{PFA}} \) (dotted line, Fig. 1D). Non-injected or H2O-injected oocytes showed either no response to PFA or a consistently smaller change in holding current for holding potentials <0 mV, (upper, Fig. 1D). Thus, the scatter in the \( I_{\text{PFA}} - I_{\text{P}} \) correlation most likely arises from contamination from endogenous responses to either P, or PFA.41,45 For \( V > 0 \text{ mV} \), control (non-injected or H2O injected) oocytes sometimes also show an endogenous response to PFA, which resembles the response of injected oocytes in the same voltage range (Fig. 1D) (see below).
from which we concluded that \( -I_{\text{PFA}} \) is a measure of the permeation of Na\(^+\) ions through the transporter. Furthermore, the dependence of \( -I_{\text{PFA}} \) on external [Na\(^+\)] shows Michaelian kinetics (apparent \( K_m = 128 \) mM), which suggests that one Na\(^+\) ion interacts with the carrier.\(^{41}\) Taken together, these findings offer compelling evidence that the underlying transport mechanism is more akin to that of a uniport class of carrier rather than a channel.

Under the tacit assumption that \( -I_{\text{PFA}} \) reflects the inhibition of an intrinsic transport-independent leak mode of NaPi-IIa, we postulated an electroneutral Na\(^+\) translocation partial reaction (2a\(\Rightarrow\)7a) takes place in the absence of Pi (Fig. 1A). The leak and cotransport modes share common partial reactions (empty carrier and Na\(^+\) binding/ debinding on the external and internal faces of the membrane) and this model predicts that at saturating \( P_i \) when the forward partial reaction 2b\(\Rightarrow\)3 is favored, the leak mode will be inactive. When PFA is bound, the protein occupies a unique blocked conformation (\(^3\)). This is similar to schemes proposed for the Na\(^+\) glucose cotransporter SGLT1 \(^{18,46}\). Importantly, if the leak and cotransport modes are mutually exclusive, the true baseline for quantitating the electrogenic activity of the cotransport mode for saturating \( P_i (I_{\text{Cor}}) \) is given by the holding current in the presence of PFA and not the holding current in the control solution (Fig. 1B).

In this model, the rate of translocation of Na\(^+\) via the leak pathway is also predicted to be very slow. We have estimated the turnover rates for the cotransport cycle of electronegic SLC34 proteins to lie in the range 4–13 s\(^{-1}\) \(^{47}\). Therefore, if one net charge is translocated in the leak mode, the magnitude of \( I_{\text{PFA}} \) suggests that the leak turnover rate will be <1 s\(^{-1}\).

The carrier mechanism for the leak mode has gained further support from a study on the temperature dependence of \( I_{\text{Pi}} \) and \( I_{\text{PFA}} \) of the flounder NaPi-IIb isoformal, which was chosen because of its large transport activity, up to 5-fold greater than mammalian isoforms.\(^{40}\) As illustrated in Figure 2A, both \( I_{\text{Pi}} \) and \( I_{\text{PFA}} \) show strong temperature dependencies that when fit with the Arrhenius equation yield activation energies (\( E_a \)) at -100 mV of -16 kcal mol\(^{-1}\) and -11 kcal mol\(^{-1}\) for \( I_{\text{Pi}} \) and \( I_{\text{PFA}} \), respectively (Fig. 2B). The estimate of \( E_a \) for \( I_{\text{PFA}} \) is consistent with that expected for a carrier type mechanism involving large conformational changes, rather than a channel. The latter should have an activation energy closer to that for free diffusion of ions (e.g., reviewed in ref. 48), for example, as reported for the Cl\(^-\) leak of GAT4.\(^{5} \) The activation energy for \( I_{\text{PFA}} \) is the same as that reported for the H\(^+\)-mediated leak of the divalent metal ion transporter DMT1,\(^{49}\) but significantly less than 26 kcal mol\(^{-1}\) reported for the Li\(^+\)-activated leak of GAT1,\(^{21}\) and 21 kcal mol\(^{-1}\) for the Na\(^+\)-activated leak of SGLT1.\(^{50}\) These data show that among members of different solute carrier families there is varying complexity of conformation changes that accompany the uncoupled leak mode. Furthermore, we found that \( E_a \) for both cotransport and leak modes is only weakly sensitive to membrane potential (Fig. 2B), which supports the assumption that the rate-limiting substrate translocation partial reactions for each mode (2a\(\Rightarrow\)7a; 4\(\Rightarrow\)5) are electroneutral.\(^{51}\) Analysis of the temperature dependence of the partial reactions associated with the empty carrier and lumped Na\(^+\) binding interactions, which are manifest experimentally as presteady-state charge movements (see below), also revealed activation energies >10 kcal mol\(^{-1}\).\(^{51}\) It is therefore conceivable that the temperature dependence of \( I_{\text{PFA}} \) reflects molecular rearrangements associated with the empty carrier and Na\(^+\) binding events and not translocation per se.

In other studies, we have documented the insensitivity of \( I_{\text{PFA}} \) to changes in external pH.\(^{45,52}\) As illustrated in Figure 3A, when the external medium is acidified, \( I_{\text{PFA}} \) at all potentials decreases, in part due to the reduced availability of the preferred divalent \( P_i \) and also because protons interact with partial reactions in the transport cycle.\(^{39,45}\) It is noteworthy that at pH 5, \( I_{\text{P}i} \) closely matches \( I_{\text{PFA}} \) at pH 7.4 (Fig. 3B), which suggests that there is a progressive pH-dependent shift from the cotransport mode to the leak mode. Moreover, these data indicate that even in its monovalent form at pH 5.0, \( P_i \) can interact with the carrier to inhibit both leak and

\[ E_a = \frac{R}{T} \log \frac{I_{PFA}}{I_{Pi}} \]
cotransport modes.\textsuperscript{52} The robust nature of \(I_{\text{PFA}}\) is also underscored by its insensitivity to the arginine modifying reagent, phenolglyoxal, which typically reduces \(I_p\) by 50%.\textsuperscript{53}

**Leak and electrogenicity go hand in hand in SLC34 proteins.** Although PFA inhibits Na\(^+\)-coupled \(P_i\) cotransport mediated by both electrogenic and electroneutral SLC34 isoforms (Ghezzi C and Stange G, unpublished data), a PFA-dependent change in holding current is only detectable in electroneutral NaPi-Ila/b isoforms (Fig. 4A and B). This suggests that the putative Na\(^+\)-leak pathway is absent in NaPi-IIc. Evidence to support this conclusion comes from studies on the WT NaPi-IIc and the WT NaPi-IIc backbone engineered to restore electrogenicity.

First, WT NaPi-IIc is proposed to lack a Na\(^+\)-binding site within the transmembrane electric field, consistent with its Na\(^+\):\(P_i\) stoichiometry of 2:1,\textsuperscript{40} (lower, Fig. 4B). Binding of one Na\(^+\) ion at this site would correspond to the 1\(^{\text{st}}\) Na\(^+\) interaction of the transport cycle (1\(\leftrightarrow\)2a) (Fig. 1A). For electrogenic NaPi-Ila/b, voltage steps induce presteady-state charge movements in 0 mM \(P_i\). These are considered to originate in part from the movement of one Na\(^+\) ion per transporter with the transmembrane electric field, as well as the movement of mobile charges associated with the empty carrier, observed in the absence of external Na\(^+\).\textsuperscript{42,43} As neither component of charge movement is detectable in oocytes that express functioning NaPi-IIc, this may indicate that in the electroneutral transport cycle, partial reaction 1\(\leftrightarrow\)2a is absent, and the reorientation of the empty carrier is electroneutral. Moreover, voltage clamping NaPi-IIc oocytes at different potentials does not alter the rate \(P_i\) uptake, which indicates that the electroneutral isoform lacks voltage-dependent partial reactions in its transport cycle.\textsuperscript{40}

Second, by comparing the sequences of the electrogenic and electroneutral isoforms, we identified a critical three amino acid motif conserved in all electrogenic isoforms that includes an aspartic acid (Fig. 6), which is absent in all electroneutral isoforms. When the residues comprising this motif, are substituted at the equivalent sites in the electroneutral backbone, the resulting mutant (AAD-IIc) displays electrogenic behavior (Fig. 4A). One net charge is transported per \(P_i\) (Fig. 4B, upper) and the Na\(^+\):\(P_i\) stoichiometry of cotransport is increased from 2:1 to 3:1 (Fig. 4B, lower).\textsuperscript{40} Moreover, presteady-state charge movements are detectable, both in the presence and absence of external Na\(^+\) (data not shown). The former finding is consistent with the re-establishment of a Na\(^+\) binding site within the transmembrane field.\textsuperscript{40} The latter finding indicates that the effective mobile charge, which confers voltage dependence to the empty carrier partial reaction (Fig. 1A), is present in AAD-IIc, possibly contributed by the substituted Asp-224. Application of PFA induces an upward deflection of the steady-state holding current (arrowed, Fig. 4A) similar to that observed for the WT electrogenic isoforms, however the \(I-V\) relationship (Fig. 4C) reveals a weaker voltage dependence for both \(I_p\) and \(I_{\text{PFA}}\) compared to the WT over the same voltage range. Thus, the substituted motif is essential, but not exclusively involved in determining the voltage dependence.\textsuperscript{40}

Like the WT, for AAD-IIc the magnitude of \(I_{\text{PFA}}\) correlates with \(I_p\) (Fig. 4D), which supports our view that \(I_{\text{PFA}}\) reflects electrogenic activity intrinsic to the expressed protein.

In summary, these findings offer compelling evidence that the presence of a leak current and the electrogenic interaction of one Na\(^+\) ion with the empty carrier are not coincidental. As this Na\(^+\) interaction is absent in the electroneutral isoform, the leak current displayed by electrogenic isoforms seems to be a direct consequence of their electrogenic transport kinetics.

**Anion replacement experiments.** The small magnitude of \(I_{\text{PFA}}\) and potential for contamination by endogenous currents that are also modulated by the specific substrates of the SLC34 family proteins

![Figure 4](image)
has prevented its full characterization over a wide range of experimental conditions, particularly for membrane potentials close to 0 mV, unless cells with high expression (I_p) are selected. One feature of I_PFA that was not fully resolved in the above studies was that the PFA-inhibitable leak reflects the activity of a Na+-dependent conductance, equilibrium thermodynamics predicts that its reversal potential (E_r) should be given by the Nernst potential. For example, if we assume a typical oocyte cytosolic [Na+] < 10 mM (reviewed in ref. 54), with an external [Na+] = 100 mM, E_r > +60 mV. In practice, we consistently document E_r in the range -10 to -30 mV (for example see Fig. 1D), which might be expected for a Cl- conductance and suggests that PFA inhibits more than one permeating ion species. Interestingly, the reversal potential for I_PFA was similar to that of phlorizin-inhibitable leak current of Na+-coupled glucose transporter (SGLT-1) (see Fig. 5C), which was shown to be dependent on internal sugar.  

Initially, we therefore adopted a similar strategy for simulations using the kinetic model for NaPi-IIa/b, by assuming a finite internal P_i (reviewed in refs. 45 and 53).

Experiments in which we replaced ~90% of external Cl- with other anions have now shed new light on the origin of the negative E_r. Substitution with the large polyatomic anions like morpholineethanesulfonate (MES) (upper, Fig. 5A) and glucuronate (lower, Fig. 5A) cause a depolarizing shift of E_r and suppression of I_PFA with external Cl- > +60 mV. In practice, we observe only a small change in the I_PFA when Cl- is removed from the external medium. Moreover, a comparison of the responses of non-injected or H2O-injected oocytes to PFA indicates that it suppresses an endogenous outward rectifying current that varies considerably between oocyte batches and according to the number of days after retrieval from the ovary. This endogenous current, which can be larger than the transporter-associated leak could therefore account for the negative E_r in NaPi-IIa/b expressing oocytes. In contrast, for V < 0 mV we observe only a small change in the I_PFA when Cl- is removed from the external medium. Moreover, I_PFA was little affected when oocytes were dialysed in a low Cl- medium to reduce the internal [Cl-] (reviewed in ref. 10) compared to non-dialysed oocytes, thereby confirming that outward movement of Cl- ions does not contribute significantly for hyperpolarizing potentials. With the notable exception of gluconate, equimolar replacement of 100 mM external chloride with a selection of anions...
indicates that Cl- ions can interact with SGLT1 and alter its voltage dependent cotransport mode current, as previously reported and behavior observed for NaPi-IIa/b expressing oocytes from the same and no shift in the reversal potential (right, Fig. 5C) unlike the

107 mM Cl-, as illustrated in Figure 5C for the flounder NaPi-IId (left) and rat NaPi-IIa (center). These isoforms show characteristically different voltage dependencies for I_{PFA} (1 mM) (e.g., Forster 2006): for hyperpolarizing potentials down to -120 mV, rat NaPi-IIa shows no rate-limiting behavior compared with the flounder NaPi-IId. The similarity of the voltage dependence of I_{PFA} for each isoform supports our notion that the leak and cotransport modes share common voltage dependent processes (Fig. 1A). Moreover, in both cases, the reduced I_{PFA} in gluconate superfusion appears as a direct consequence of the subtraction procedure used to eliminate endogenous currents in which the leak is, in effect, subtracted from the response to P_i. The increase in leak with gluconate superfusion appears to be a unique property of NaPi-IIa/b proteins expressed in Xenopus oocytes. We examined the effect of gluconate substitution on the intrinsic leak of SGLT1, using phlorizin to block its uncoupled leak. Here, we documented no significant difference between the phlorizin-dependent currents in 107 mM Cl- or with 100 mM gluconate and no shift in the reversal potential (right, Fig. 5C) unlike the behavior observed for NaPi-IIa/b expressing oocytes from the same donor frog. Gluconate replacement slightly reduced the α-MNG-dependent cotransport mode current, as previously reported and indicates that Cl- ions can interact with SGLT1 and alter its voltage dependent kinetics. However, the negative E_C for SGLT1 cannot be explained simply by the involvement of Cl- ions. Finally, we also confirmed that PFA-inhibitable current measured with gluconate replacement has the properties of Na+-dependent conductance (Fig. 5D). Equimolar replacement of Na+ with Li+, which does not drive transport in SLC34 transporters, shifted the E_C of I_{PFA} according to the Nernstian relationship with slopes of 54.9 ± 6.5 mV/log[Na+] (rat NaPi-IIa, n = 3) and 51.9 ± 2.4 mV/log[Na+] (human NaPi-IIa, n = 3) (Forster IC, unpublished data). These data strongly suggest that the main permeating ion in the leak mode is Na+, in agreement with our earlier findings.

The mechanism by which gluconate increases the PFA-inhibitable leak of NaPi-IIa/b is unclear and will require further investigation. As gluconate is a known chelator of divalent cations, we confirmed that the appropriate reduction of free [Ca^{2+}] and [Mg^{2+}] in the external buffer, to simulate chelation by gluconate, was not the underlying cause. The main effect of gluconate substitution on NaPi-IIa/b kinetics is to increase the leak current, most likely by altering the rate of the Na+-translocation step 2a↔7a (Fig. 1A), whereas the predicted cotransport activity is only marginally affected. In this respect, the behaviour of NaPi-IIa/b with gluconate superfusion supports the notion that P_i induces a switch from the leak to cotransport cycle.

New insights from structure-function studies- evidence that leak and cotransport share the same pathway. The current secondary topology of SLC34 proteins (Fig. 6) is based on prediction algorithms and structure-function studies (reviewed in refs. 37 and 43). The functional unit of NaPi-IIa is a monomer, however there is also evidence that it may dimerize in the membrane. Each monomer comprises a core backbone of eight transmembrane domains interspersed with two repeat regions in the C- and N-terminal halves of the protein. We propose that they form two reentrant loops that associate to constitute the transport pathway. Cysteine scanning studies have confirmed that parts of loop ECL-3 and ICL-1 are accessible from the external and internal sides of the membrane, respectively. In ECL-3, evidence of a partial α-helical structure was obtained from cysteine scanning. However no detailed topological features have been identified for the outwardly oriented ICL-1. As no 3-D structure for members of the SLC34 family or its bacterial homologs currently exist and there is no homology with available 3-D structures of other Na+-dependent cotransporters, this functional assignment of

![Image](521x738)

**Figure 6.** Secondary topology of SLC34 family proteins. Topology is based on the rat NaPi-IIa sequence and predicted according to (81). One essential cysteine bridge (black, dashed line) links the N- and C-terminal halves of the protein and there are two N-glycosylation sites in the large extracellular loop. The two re-entrant loops, proposed to form the transport pathway, are shaded (light blue) contain repeat residues (pink). Selected sites, relevant to leak and cotransport modes, which have been identified from structure-function studies, in which either the leak mode or cotransport mode or both are affected by MTS incubation are indicated as follows: blue: leak unchanged; green: cotransport fully suppressed, leak unchanged; yellow: cotransport fully suppressed, leak increased. In addition, sites critical for electrogenticity are shown (red). © Virkki et al., adapted from Figure 3 originally published in AJP-renal Physiol 2007; 293:643–54.
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Figure 7. Structure function studies separate leak from cotransport activity. (A) S460C mutant. Representative current recording at -50 mV showing response to Pi (1 mM) and PFA (3 mM) under control conditions. The oocyte was then exposed to the methanethiosulfonate reagent MTSEA (1 mM) for 3 minutes and then the measurement was repeated. © Lambert et al., adapted from Figure 6 originally published in The J Gen Physiol 1999; 114:637–51. Inset shows I-V data for S460C and establishes that I_{pi} after and I_{PFA} before and after MTSEA exposure are almost identical over a wide range of test potentials. Data points normalised to I_{pi} at -100 mV before MTSEA exposure (n = 4). Filled symbols: control; empty symbols: after MTSEA exposure; squares: I_{PFA}; circles: I_{Cot}. (Forster IC, unpublished data). (B) A453C mutant. Representative recording at -50 mV showing response to Pi (1 mM) and PFA (3 mM) under control conditions and after exposure to MTSEA for 3 min at the indicated concentrations. Only the baseline that immediately preceded application and washout of the test substrates is shown. The dashed line indicates holding current reached during control PFA application. Continuous line indicates initial holding current in control superfuse. No external adjustment of current offset was made during the recording. After 100 μM MTSEA application there is little change in the responses to Pi and PFA. © Lambert et al., adapted from Figure 3 originally published in The J Gen Physiol 2001; 117:533–46. (C) The double mutant A203C-S460C. Representative recording at -50 mV showing response to Pi (1 mM) and PFA (3 mM) under control conditions and after exposure to MTSEA (1 mM for 3 min) for mutant A203C (upper) and S460C (lower) and A203C-S460C. Inset shows the reciprocity of transport mode activity as A203CS460C is progressively modified using MTS reagents MTSEA (squares) and MTSES (triangles). Loss of cotransport function (filled symbols) and gain in leak (open symbols) follow reciprocal time courses suggesting that the two processes are linked. Continuous (MTSEA) and dotted (MTSES) lines are fits to the data with a single exponential function to give modification rates for MTSEA (leak gain: 7.3 x 10^{-4} s^{-1} μM^{-1}; cotransport loss: 7.2 x 10^{-4} s^{-1} μM^{-1}) and MTSES (leak gain: 1.1 x 10^{-3} s^{-1} μM^{-1}; cotransport loss: 1.2 x 10^{-3} s^{-1} μM^{-1}). Each data point represents mean ± SEM (n = 6). (For further details see ref. 29.) © Kohler et al., adapted from Figures 2 and 6 originally published in The J Gen Physiol 2002; 120:693–705.

Insights into the nature and localization of the leak pathway for SLC34 proteins have come from structure-function studies in which cysteine residues are substituted at functionally important sites. This involves determining accessibility by methanethiosulfonate (MTS) reagents of the novel cysteine and characterizing the effect of the cyc-substitution and MTS-modification on the I_{pi} and I_{PFA}.\textsuperscript{26,29,64,65} In these studies, a number of mutants show full suppression of cotransport activity after MTS modification, which confirms their accessibility. These can be further distinguished according to the effect cyc-modification has on the PFA-inhibitable leak: constructs in which the leak is unchanged after cyc-modification and those in which the leak activity is increased.

Mutant S460C exemplifies the first type of behavior, which has a Ser-Cys substitution at a site in the C-terminal end of reentrant loop ECL-3 (Fig. 6). The substitution alone does not alter the basic kinetic properties compared to the WT.\textsuperscript{26} However, after exposure to MTS reagents that increase the residue bulk and alter the charge at the substitution site, cotransport activity is inhibited, and the electrogenic responses to Pi (1 mM) and PFA (3 mM) are identical (Fig. 7A). This behavior can readily be understood in terms of the kinetic scheme (Fig. 1A), whereby Pi and PFA compete for the same binding site, thus blocking the leak mode. In the case of S460C, after MTS exposure, the substrate binding partial reactions for binding of Na\textsuperscript{+} (1-2a, 2a-2b) and Pi (2b-3) still occur, however subsequent partial reactions in the transport cycle are excluded because the modified cysteine restricts the possible conformational states the protein can occupy. With respect to the reentrant segments remains speculative. Nevertheless, the repeat regions are also conserved in the prokaryotic homolog from Vibrio cholerae.\textsuperscript{33,59} Moreover, recent 3-D structures of Na\textsuperscript{+}-driven cotransporters\textsuperscript{60-63} have highlighted the importance of the inverted repeat structure that defines the substrate binding region and which could well apply to SLC34 proteins.
the leak mode, state 3* (PFA bound) and state 3 (P bound) are therefore indistinguishable, in agreement with the equivalence of \( I_{pK} \) and \( I_{PFA} \) over a wide range of test potentials (inset, Fig. 7A). At least one site has also been identified that shows similar behavior to S460C in the predicted reentrant loop ICL-1.28 The cotransport activity of this mutant N199C (Fig. 6) is modified using the partially membrane permeable reagent MTSEA, whereas the impermeable MTSET has no effect. In addition to providing confirmation of the predicted topology, this finding is a first, albeit indirect hint that these reentrant loops might contribute to a common transport pathway for both the leak and cotransport modes. The critical importance of Asn-199 was demonstrated by making other substitutions at this site. Mutants in which the native Asn is substituted with the amide derivative of glutamate (Gln) or other basic or acidic amino acids (Asp, His, Arg) show only leak (PFA response), whereas substituting with Cys, Ala and Thr is tolerated and the corresponding mutants show both leak and cotransport activity.28

Mutant A453C also with a cis-substitution in ECL-3, exemplifies the second type of behavior. MTSEA treatment induces a large Na+-dependent leak that, like S460C, is equally suppressed by both PFA and \( P_i \) (Fig. 7B).27 As the MTSEA concentration is increased, a progressively greater fraction of transporters is modified and \( I_{P_i} \) changes from the initial downward deflection, characteristic of cotransport, to an upward deflection, which represents inhibition of the leak current. Note that the holding current reached during PFA application remains the same and corresponds to \( I_{Endog} \) (Fig. 1A). In the GABA transporter GAT1 a similar phenotype of increased leak after MTS modification of Cys-74 in the first reentrant loop region33 which, according to the 3-D structure of the bacterial homolog LeuT\(_{Asa}\), forms part of the substrate binding domain.63,66

These findings provide compelling evidence for an interaction between ICL-1 and ECL-3, which was further investigated by examining the effect on the Cys-Ser substitution at site 460 by making a second cis substitution at site Ala-203 in ICL-1.29 The single mutant (A203C) exhibits a significantly larger \( I_{P_{FA}} \) compared with the WT28 (Fig. 7C). As expected, the double mutant (A203C-S460C) displays the phenotype of A203C, which indicates that the Cys-460 does itself alter the electrogenic activity. However, after exposure to MTSEA, the leak increases and \( P_i \) induces the same change in holding current as PFA,29 thus recapitulating the phenotype observed for Ala-Cys substitution in ECL3 (Fig. 7B). Based on the behavior of this and other mutants, our current view is that the leak and cotransport pathways share common structural elements. Whether one or both novel Cys in A203C-S460C are modified remains to be determined, however we currently favor a steric interaction between Cys-203 and Cys-460, in which only Cys-460 is modified, based on the behavior of other substitutions at these sites and a comparison of the rates of modification (Fig. 7C, inset).29 The A203C-S460C double mutant also provides a useful tool to demonstrate the close interrelationship between leak and cotransport modes. The progressive growth in leak and decrease in cotransport activity over the time course of cis-modification is reciprocal (Fig. 7C, inset). This is the behavior expected of two populations of transporters: those that are modified and mediate only leak and those that are unmodified and can still mediate cotransport.29

**Transport kinetics and leak.** Transport-independent leak currents can introduce a source of error and lead to data misinterpretation when characterizing the transport kinetics of electrogenic carriers. As noted above, some WT cotransporters display leak currents that are comparable in magnitude to the substrate-induced change in holding current, (e.g., NIS,15 and SVCT1,2, [ref. 16]). If our model of mutual exclusivity of the leak and cotransport modes is valid also for other electrogenic carriers, large leak currents should be taken into account when deriving phenomenological kinetic parameters, such as apparent substrate affinities. Errors will arise when the substrate-induced currents are measured relative to the leak current (i.e., the baseline current in the absence of substrate, see Fig. 1A), which would substantially underestimate the magnitude of the cotransport mode current.43 At non-saturating \([P_i]\), the overall electrogenic activity will be contributed by both leak and cotransport modes: depending on the probability of occupancy of the associated states, a fraction of the transporters will translocate charge in the leak mode and others in the cotransport mode.

For cotransporters like NaPi-IIa/b, and SGLT1, where the leak typically accounts for ≤20% of substrate induced current, this error is small. For SGLT1, it was estimated that the leak contribution to electrogenic activity is negligible for glucose concentrations well below the apparent affinity for glucose cotransport18 and a similar analysis has yet to be undertaken for NaPi-IIa/b. However, for NaPi-IIa/b, the error can become larger under specific conditions, such as low pH (Fig. 2A) or gluconate perfusion (Fig. 5C), where the relative contribution of the two transport modes to electrogenic activity changes significantly. This could account a significant deviation in stoichiometry of NaPi-IIa/b observed at pH < 6.8 determined by simultaneous uptake and charge measurements on single oocytes. Here, the transported charge is quantified by integrating the steady-state response to substrate (e.g., \( I_{pK} \) in Fig. 1B), using the control solution as baseline (reviewed in refs. 15, 38 and 67). At pH 7.4 and 6.8, the Q:P\(_i\) ratio for rat NaPi-IIa expressing oocytes is 1.0,38 whereas at pH 6.2, where \( I_{pK} \) is significantly smaller but the leak is unchanged, Q:P\(_i\) = 0.5 ± 0.1 (n = 13) (Forster IC, Loo DDF, Eskandari S, unpublished experiments). It may also explain the non-integer estimate of stoichiometry reported for the Na\(^+\)-coupled iodide cotransporter (NIS).15

**Conclusions**

The physiological significance the NaPi-IIa/b leak is unclear: for example, along the length of the renal proximal tubule, NaPi-IIa in the brush border membrane is mostly exposed to \( P_i \) levels that exceed \( K^m \) and therefore we would not expect the substrate-independent leak to contribute to a significant inward Na\(^+\) flux. However, in the context of structure-function investigations, the leak mode offers a useful phenotype for gaining insight into transport mechanisms. Whereas many transporters exhibit channel-like (gated pore) behavior with complex permeation phenotypes (see Table 1), the Na\(^+\)-dependent leak current of SLC34 proteins appears to result from the intrinsic translocation of one charge per transport cycle and is specific for Na\(^+\). Although we cannot exclude a leak mechanism in which Na\(^+\) itself gates a thermodynamically uncoupled Na\(^+\)-selective pore, the evidence we have obtained so far from WT and mutant constructs is consistent with the leak being intimately associated with the electrogenic interaction of a single Na\(^+\) ion, and its subsequent translocation via a unipor mechanism. In this respect, the term “uncoupled” leak should be used with reservation. Importantly, the leak and cotransport modes of NaPi-IIa/b appear to be mutually
exclusive: the behavior of the Cys mutants provides strong evidence that the leak mode is fully suppressed when substrate is bound. Here, we assume that the mutant behavior reflects the transitions between a subset of conformational states also occupied in the WT. Therefore, according to the transport scheme (Fig. 1A), for a given external [Na\(^+\)], the concentration of P\(^-\) will determine whether the protein translocates only Na\(^+\) ions via the leak loop (transition 2→7), or binds P\(^-\) and cotransports 3 Na\(^+\) ions and P\(^-\) (transition 4→8→5), with the same Na\(^+\) ion common to both modes.

Finally, we note that our data are based exclusively on experiments with intact oocytes and constant intracellular environment. To test the validity of the alternating access model implied by the kinetic scheme of Figure 1A, further investigations would benefit from the cut-open oocyte technique, to allow access to the intracellular medium (reviewed in ref. 68).

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