



Year: 2013

Conferring electrogenic to the electroneutral phosphate cotransporter NaPi-IIc (SLC34A3) reveals an internal cation release step

Patti, Monica ; Ghezzi, Chiara ; Forster, Ian C

Abstract: The SLC34 family of Na(+)-dependent inorganic phosphate cotransporters comprises two electrogenic isoforms (NaPi-IIa, NaPi-IIb) and an electroneutral isoform (NaPi-IIc). Both fulfill essential physiological roles in mammalian phosphate homeostasis. By substitution of three conserved amino acids, found in all electrogenic isoforms, at corresponding sites in NaPi-IIc, electrogenicity was re-established and the Na(+)/P_i stoichiometry increased from 2:1 to 3:1. However, this engineered electrogenic construct (AAD-IIc) had a reduced apparent P_i affinity and different presteady-state kinetics from the wild-type NaPi-IIa/b. We investigated AAD-IIc using electrophysiology and voltage clamp fluorometry to elucidate the compromised behavior. The activation energy for cotransport was threefold higher than for NaPi-IIc and 1.5-fold higher than for NaPi-IIa and the temperature dependence of presteady-state charge displacements suggested that the large activation energy was associated with the empty carrier reorientation. AAD-IIc shows a weak interaction of external Na(+) ions with the electric field, and thus retains the electroneutral cooperative interaction of two Na(+) ions preceding external P_i binding of NaPi-IIc. Most of the presteady-state charge movement was accounted for by the empty carrier (in the absence of external P_i), and the cytosolic release of one Na(+) ion (in the presence of P_i). Simulations using a kinetic model recapitulated the presteady-state and steady-state behavior and allowed identification of two critical partial reactions: the final release of Na(+) to the cytosol and external P_i binding. Fluorometric recordings from AAD-IIc mutants with Cys substituted at functionally important sites established that AAD-IIc undergoes substrate- and voltage-dependent conformational changes that correlated qualitatively with its presteady-state kinetics.

DOI: <https://doi.org/10.1007/s00424-013-1261-9>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-84160>

Journal Article

Accepted Version

Originally published at:

Patti, Monica; Ghezzi, Chiara; Forster, Ian C (2013). Conferring electrogenic to the electroneutral phosphate cotransporter NaPi-IIc (SLC34A3) reveals an internal cation release step. *Pflügers Archiv : European Journal of Physiology*, 465(9):1261-1279.

DOI: <https://doi.org/10.1007/s00424-013-1261-9>

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Abstract

1 The SLC34 family of Na⁺-dependent inorganic phosphate cotransporters comprises two electrogenic isoforms
2 (NaPi-IIa, NaPi-IIb) and an electroneutral isoform (NaPi-IIc). Both fulfil essential physiological roles in
3 mammalian phosphate homeostasis. By substitution of 3 conserved amino acids, found in all electrogenic
4 isoforms, at corresponding sites in NaPi-IIc, electrogenicity was re-established and the Na⁺:P_i stoichiometry
5 increased from 2:1 to 3:1. However, this engineered electrogenic construct (AAD-IIc) had a reduced apparent P_i
6 affinity and different presteady-state kinetics from the wild-type NaPi-IIa/b. We investigated AAD-IIc using
7 electrophysiology and voltage clamp fluorometry to elucidate the compromised behavior. The activation energy
8 for cotransport was 3-fold higher than for NaPi-IIc and 1.5-fold higher than for NaPi-IIa and the temperature
9 dependence of presteady-state charge displacements suggested that the large activation energy was associated
10 with the empty carrier reorientation. AAD-IIc shows a weak interaction of external Na⁺ ions with the electric
11 field, and thus retains the electroneutral cooperative interaction of 2 Na⁺ ions preceding external P_i binding of
12 NaPi-IIc. Most of the presteady-state charge movement was accounted for by the empty carrier (in the absence
13 of external P_i), and the cytosolic release of one Na⁺ ion (in the presence of P_i). Simulations using a kinetic model
14 recapitulated the presteady-state and steady-state behavior and allowed identification of two critical partial
15 reactions: the final release of Na⁺ to the cytosol and external P_i binding. Fluorometric recordings from AAD-IIc
16 mutants with Cys substituted at functionally important sites established that AAD-IIc undergoes substrate- and
17 voltage-dependent conformational changes that correlated qualitatively with its presteady-state kinetics.
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33 Key words: phosphate cotransport, electrophysiology, voltage clamp fluorometry, presteady-state, steady-state,
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Introduction

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2 Type II sodium/phosphate cotransporters (NaPi-II) catalyse transport of inorganic phosphate (P_i) using
3 the transmembrane Na^+ electrochemical gradient. They belong to the SLC34 family of solute carriers
4 (www.bioparadigms.com) (15) that preferentially transport divalent P_i (HPO_4^{2-}) (17). The family comprises 3
5 isoforms to which all currently identified members have been assigned according to their genetic pedigree,
6 molecular features and tissue localization: the electrogenic NaPi-IIa and electroneutral NaPi-IIc are localized
7 exclusively in the renal proximal tubule and the electrogenic NaPi-IIb is expressed in non-renal, epithelial-like
8 tissues. The difference between the electrogenic and electroneutral isoforms is reflected in their $Na^+:P_i$
9 cotransport stoichiometries: NaPi-IIa,b (SLC34A1, A2) have a 3:1 stoichiometry (17) and translocate one net
10 charge per transport cycle; NaPi-IIc (SLC34A3) has a 2:1 stoichiometry with no detectable net charge
11 translocation. The electrogenic and electroneutral cotransport mechanisms can be understood in terms of a
12 kinetic scheme (Fig 1A) that describes their respective transport cycles as an ordered sequence of partial
13 reactions, some of which have been identified experimentally. For example, electrogenicity can be accounted for
14 by assigning charge displacement to the empty carrier ($0 \leftrightarrow 1$) together with extra- and intracellular interactions of
15 one Na^+ ion ($1 \leftrightarrow 2$, $0 \leftrightarrow 9$) (2, 13, 19). This cation is postulated to account for the uncoupled leak (2). To
16 reconcile this model with electroneutral cotransport mediated by SLC34A3 (NaPi-IIc), the scheme was modified
17 (*shaded area*, Fig 1A), based on the interpretation of fluorometric assays (20). For NaPi-IIc, 2 Na^+ ions are
18 assumed to interact with the protein before P_i , but unlike NaPi-IIa/b, only one of these cations contributes to net
19 transport. We postulated that the first Na^+ ion to interact with NaPi-IIc acts as a “catalytic activator” for
20 cotransport (20) that gives rise to cooperative binding of a 2nd Na^+ ion before P_i binds ($2 \leftrightarrow 3$). This 1st Na^+ ion
21 would remain bound during the cotransport cycle, but contributes neither to net coupled transport nor leak,
22 which in the case of NaPi-IIc is undetectable (4). Thus, for the electrogenic isoforms both leak and cotransport
23 modes involve the translocation of one net positive charge between the external and internal milieus, and both
24 involve an electroneutral intrinsic translocation of substrate (transitions $2 \leftrightarrow 9$ and $5 \leftrightarrow 6$, respectively).
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27 Apart from electrogenicity and leak, other kinetic properties (apparent substrate affinities, pH
28 dependence) are essentially the same for the electrogenic and electroneutral isoforms (13, 31, 33). These
29 common functional properties accord with the finding that SLC34 proteins display an overall amino acid
30 similarity of $\approx 57\%$. This is even greater in the predicted transmembrane domain regions (36) and therefore all
31 isoforms most likely share a common 3-D structure. The current lack of a 3-D structure and apparent absence of
32 homology with membrane transport proteins with resolved structures has necessitated using indirect approaches
33 (mutagenesis, cysteine scanning, crosslinking) to determine the structure-function relationships (16, 19, 20).
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36 It is noteworthy that small differences in the amino acid sequence in SLC34 proteins can lead to
37 significant functional changes in pH sensitivity (9) and electrogenicity (4). In the latter case, we identified a trio
38 of amino acids (Ala-218, Ala-220, Asp-224, mouse NaPi-IIa sequence numbering), located in a putative linker
39 region between two predicted α -helical transmembrane domains (TMD4 & 5, Fig 1 B), which were shown to be
40 critical for electrogenic behavior. A comparison of the sequences of the electrogenic isoforms from a number of
41 species reveals that the Ala-Ala-Asp trio is conserved. In the electroneutral isoforms these are replaced with a
42 Ser-Ser-Gly trio (Fig 1 B, *inset*). If the Ala-Ala-Asp trio was substituted at the equivalent sites in the
43 electroneutral NaPi-IIc (Ser-189, Ser-191, Gly-195) electrogenicity was re-established, as evidenced by robust
44 P_i -induced steady-state currents, presteady-state relaxations and the predicted 3:1 stoichiometry (4), all of which
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1 are hallmarks of electrogenic SLC34 proteins. Moreover, the replacement of the critical aspartic acid in NaPi-IIa
2 with a glycine resulted in electroneutral cotransport (32). However, the molecular engineering of NaPi-IIc
3 resulted in compromised cotransport kinetics. The modified protein (hereafter referred to as AAD-IIc) showed a
4 >200-fold reduced apparent affinity for P_i : the Michaelis constant ($K_{0.5}^{P_i}$) was >1 mM (compared with ≈ 0.06 mM
5 for NaPi-IIc), whereas for Na^+ activation (1 mM P_i), the apparent affinity constant ($K_{0.5}^{Na}$) ≈ 80 mM was only
6 marginally affected (≈ 40 mM for NaPi-IIc) (19). Furthermore, compared with other electrogenic isoforms, the
7 presteady-state charge movements appeared to show little dependence on external $[Na^+]$ (e.g. (2, 33)). These
8 deviations from expected behavior suggested that other differences in the respective amino acid sequences were
9 also important determinants of the AAD-IIc kinetic profile.
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11 This study focuses on the identification of partial reactions in the SLC34 cotransport cycle (Fig 1A) that
12 confer the unique behavior of AAD-IIc. By combining presteady-state analysis and voltage clamp fluorometry
13 (VCF) under conditions where the state occupancy of AAD-IIc can be readily defined, we could relate these
14 partial reactions to specific voltage-and substrate-dependent conformational changes of the protein. Our findings
15 offer new insights into how membrane voltage is transduced by cation-driven transporters and provide
16 compelling evidence that the final step in substrate release to the cytosol involves the release of a single Na^+ ion.
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Methods

Solutions and reagents

Standard extracellular solution (100Na) contained (in mM): 100 NaCl, 2 KCl, 1.0 MgCl₂, 1.8 CaCl₂, 10 HEPES, pH 7.4 adjusted with TRIS base. In Na⁺ substitution experiments NaCl was equimolarly replaced with choline Cl (100Ch) and solutions with intermediate [Na⁺] were obtained by mixing 100Na with 0Na in appropriate portions to maintain a constant molarity. P_i was added from a 1M K₂HPO₄/ KH₂PO₄ stock premixed to give pH 7.4. Modified Barth's solution for storing oocytes contained (in mM): 88 NaCl, 1 KCl, 0.41 CaCl₂, 0.82 MgSO₄, 2.5 NaHCO₃, 2 Ca(NO₃)₂, 7.5 HEPES, pH 7.5 adjusted with Tris and supplemented with 5 mg/l doxycyclin and 5 mg/l gentamicin. All standard reagents were obtained from either Sigma-Aldrich or Fluka (Buchs, Switzerland). 2-(trimethylammonium) ethylmethanethiosulfonate bromide (MTSET) and 2-aminoethyl methanethiosulfonate (MTSEA) were obtained from Toronto Research Chemicals or Biotium (USA); 2-((5(6)-tetramethylrhodamine)carboxylamino) ethyl methanethiosulfonate (MTS-TAMRA) was obtained from Biotium (USA).

Site-directed mutagenesis and cRNA preparation

cDNA encoding wild-type (WT) mouse NaPi-IIc (GenBank/EMBL/DDBJ accession n. AB054999) was subcloned into a plasmid (pT7T3D-Pac, a kind gift from K.-I. Miyamoto, Tokushima University, Tokushima, Japan). Novel cysteines were introduced using the Quickchange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA) into the AAD-IIc protein at the following sites according to the mouse NaPi-IIc sequence numbering (new mutant name in parenthesis): Gly-105 (AAD-IIc-105); Val-154 (AAD-IIc-154); Ser-155 (AAD-IIc-155); Ala-222 (AAD-IIc-222); Leu-309 (AAD-IIc-309); Ser-437 (AAD-IIc-437); Asp-440 (AAD-IIc-440) and Leu-510 (AAD-IIc-510). The sequence was verified by sequencing (Microsynth, Switzerland), linearized with *NotI* and cRNA was synthesized in the presence of Cap analog using the T7 Message Machine kit (Ambion, Inc., Austin, TX, USA).

*Expression in *Xenopus laevis* oocytes*

Female *X. laevis* frogs were purchased from *Xenopus* Express (France). Portions of ovaries were surgically removed from frogs anesthetized in MS222 (tricaine methansulphonate) and cut in small pieces. Oocytes were treated for 45 min with collagenase (crude type 1A) 1 mg/ml in 100Na solution (without Ca²⁺) in presence of 0.1 mg/ml trypsin inhibitor type III-O. Healthy stage V-VI oocytes were selected, maintained in modified Barth's solution at 16°C and injected with 10 ng of cRNA. Experiments were performed 4-7 days after injection. All animal procedures were conducted in accordance with the Swiss Cantonal and Federal legislation relating to animal experimentation.

Two-electrode voltage clamp

All voltage clamp experiments were performed using a two-electrode voltage clamp (TEC-10CX, NPI, Tamm, Germany). Oocytes were impaled with microelectrodes filled with 3 M KCl, with a typical resistance < 1MΩ (13, 18). The temperature of the recording chamber was monitored using a thermistor probe (TS-2, NPI, Tamm, Germany) placed close to the oocyte. The temperature of the recording chamber and incoming superfusate were regulated using Peltier cooling elements driven by a continuous feedback controller (TC-10, NPI Tamm, Germany). Data acquisition was performed using a 1440 Digidata (Molecular Devices Corp, USA). For recordings at constant holding potential, currents were acquired at >20 samples/s and filtered at 10 Hz. Faster sampling rates (up to 20k samples/s) were used for voltage step recordings with filtering (digital and analog) adjusted accordingly.

Steady-state currents were obtained using a protocol in which membrane voltage steps were made from the holding potential (V_h) = -60 mV, to test voltages in the range -180 mV to +80 mV in 20 mV increments. The steady-state P_i -dependent current (I_{P_i}) was obtained by subtracting control traces (in 100Na solution) from the corresponding traces in the presence of P_i . The current was measured in a region where all presteady-state relaxations were completed. Data was rejected if contaminated by endogenous Cl^- currents. Steady state P_i activation was determined by varying the P_i concentration in presence of 100Na and subtracting the respective currents in 100Na from those in 100Na+ P_i . Steady state P_i -induced currents (I_{P_i}) were fit with a form of the modified Hill Equation:

$$I_{P_i} = I_{P_i}^{\max} \left([S]^{n_H} / ([S]^{n_H} + (K_{0.5}^S)^{n_H}) \right) + K \quad (1)$$

where $[S]$ is the concentration of variable substrate (Na^+ or P_i), $I_{P_i}^{\max}$ is the maximum electrogenic activity, $K_{0.5}^S$ is the apparent affinity constant for substrate S , n_H is the Hill coefficient and K is a constant that take in account of uncoupled leak effects (10). For P_i activation, the Michaelian form of Eqn 1 was used with $n_H = 1$.

Pre-steady state relaxations were recorded using a similar voltage step protocol as for the steady-state assay with steps from $V_h = -60$ mV or 0 mV, as indicated, to test voltages in the range of -180 to +80 mV in 20 mV increments. The pulse period and period after return to the holding potential was chosen to ensure that relaxations were completed within the recording time window. Signal averaging (typically 4-fold) was used to reduce noise. Relaxations were quantified by fitting with one or two component exponential functions unless otherwise noted, commencing the fit approximately 5 ms after the ON or OFF step onset to ensure that endogenous charging components had reached a steady-state. The fits were back-extrapolated to a time point 2ms after the step onset, at which approximately 90% of the membrane was charged. The fits were numerically integrated to obtain the charge moved (Q) for a step from the holding potential to the test potential. The Q - V data were fitted with a Boltzmann function of the form given by:

$$Q = Q_{hyp} + Q_{max} / (1 + \exp(z e (V_{0.5} - V) / kT)) \quad (2)$$

where $V_{0.5}$ is the voltage at which the charge is distributed equally between two hypothetical states, z is the apparent valence of an equivalent charge that moves through the whole of the membrane field, Q_{max} is the total mobile charge available, Q_{hyp} is the charge of the hyperpolarizing limit and is a function of V_h , and e , k and T have their usual meanings.

Determination of activation energy

The Arrhenius equation (e.g.(37)) was used for determining the temperature dependence of the transport kinetics:

$$k_r = A \exp\left(-\frac{E_a}{RT}\right) \quad (3a)$$

where k_r is the reaction rate (substrate-induced current or presteady-state current relaxation rate), A is a constant, R the gas constant and E_a the activation energy (in kcal mol⁻¹). Eqn 3a can be recast in a logarithmic form:

$$\ln k_r = -(E_a/R) (1/T) + \ln A \quad (3b)$$

Linear regression analysis applied to the data plotted as $\ln k_r$ vs $1/T$ was used to estimate E_a from the slope.

³²P uptake

Oocytes (6-10 oocytes/group) were first allowed to equilibrate in 100Na solution without tracer. After aspiration of this solution, oocytes were incubated in 100Na solution containing 1mM cold P_i and ^{32}P (specific activity 10 mCi/mmol P_i). Uptake proceeded for 10 min and then oocytes were washed 3-4 times with ice-cold 100Ch solution containing 2 mM P_i , and lysed individually in 10% SDS for 1 hour before addition of scintillation cocktail. The amount of radioactivity taken up by each oocyte was measured by scintillation counting. For temperature-dependence assays, individual oocytes were incubated in the uptake medium and held at the required temperature using a water bath cooling system.

Simultaneous voltage clamp and fluorometry

The apparatus for simultaneous VCF has been described in detail elsewhere (34, 35). Cys mutants were labelled in 100Na in presence of MTS-TAMRA (0.4 mM) at room temperature for 4 min. Voltage dependent changes in fluorescence intensity (ΔF) were determined using a voltage step protocol in which the membrane voltage was stepped from $V_h = -60$ mV to test potentials ranging between -200 mV and +200 mV in 40 mV increments for a duration of 100-200 ms, and averaged over 20-64 sweeps. Fluorescence signals were analog filtered (8-pole Bessel) with a low-pass cutoff frequency chosen to optimise the signal-to-noise ratio (typically 500Hz). ΔF was measured in 100Ch, XNa (where X represents the $[Na^+]$ in mM, or 100Na +1 P_i (100 mM Na^+ +1mM P_i); each test substrate concentration application was bracketed with a control solution application (100Na) to allow for correction of a loss of fluorescence. After application of the superfusate, the oocyte was allowed to stabilize in the recording chamber for ~2 min and then fluorescence was recorded. Recordings were baseline corrected relative to the value at $V_h = -60$ mV. The data from single oocytes were corrected for photobleaching and then were normalized to the predicted maximum change in fluorescence (ΔF_{max}) recorded in 100Na and finally pooled. The ΔF -V data were described analytically by fitting with the Boltzmann equation (Eqn 2) to yield the maximum change in fluorescence intensity (F_{max}), the midpoint voltage of the ΔF -V distribution ($V_{0.5}^F$) and a slope factor z^F .

Thiol modification by MTS reagents

MTSET (or MTSEA) was prepared from dry stock in water or DMSO to give 1 M stock solution in DMSO, which was kept at -20°C until required and further diluted with water or DMSO to give 1000:1 experimental stocks before adding to 100Na solution to give a final concentration as indicated. MTSET was applied to the recording chamber with gravity feed via a 0.5-mm-diameter cannula positioned near the cell. For normal labelling, the incubation time was 3 minutes and was followed by a 1 minute wash out period. For determining the apparent accessibility, the superfusion with MTSET was conducted for 1 min followed by washout. During incubation, oocytes were kept at $V_h = -50$ mV unless otherwise noted. After a 1 min washout the current response to 1 mM P_i at $V_h = -50$ mV was again determined and the MTSET application was repeated. The fixed MTSET concentrations were chosen by pretesting to facilitate determination of the modification rate. The P_i -induced current remaining after each successive application of MTS reagent was measured and plotted as a function of the cumulative exposure time, normalized to the initial P_i -induced current. The data were fit with a single decaying exponential to determine the effective second order reaction constant using an equation of the form:

$$I_{p_i}^t = (I_{p_i}^0 - I_{p_i}^\infty) \exp(-ctk^*) + I_{p_i}^\infty \quad (4)$$

1 where $I_{P_i}^t$ is the P_i -induced current after a cumulative exposure time t , $I_{P_i}^0$ is the initial P_i -induced
2 current, $I_{P_i}^\infty$ is the P_i -induced current at $t = \infty$, c is the concentration of MTS reagent (assumed to be in excess)
3 and k^* is the effective second order rate constant e.g. (22). For some experiments as indicated, MTSEA was used
4 instead of MTSET and gave the same results.
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6 ***Software and data analysis.***
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8 Simulations of a 7-state model describing electrogenic cotransport were performed using Berkeley
9 Madonna V8.0.2a8 software (www.berkeleymadonna.com). Exponential curve fitting was performed using
10 Clampfit software (Molecular Devices Corp) and subsequent non-linear regression of data was performed using
11 GraphPad Prism version 3.02/ 4.02 for Windows, (GraphPad Software, San Diego California USA,
12 www.graphpad.com). Data points are shown as mean \pm sem. Error bars are not displayed if smaller than symbol.
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Results

Temperature dependence of steady-state and presteady-state kinetics

To identify partial reactions in the cotransport cycle that determine the unique behavior of AAD-IIC, we measured the temperature dependence of steady-state P_i -induced current (I_{P_i}) and presteady-state relaxations. The rationale for these experiments was that if one or more partial reactions were influenced by the mutagenesis, the activation energy may differ from the WT.

At all membrane potentials (V) in the test range ($-180 \text{ mV} \leq V \leq +80 \text{ mV}$), I_{P_i} (100Na, 1 mM P_i) showed a strong temperature dependence, whereby it decreased by approximately 60% for a decrease in temperature from 24 °C to 20 °C (Fig 2A). For the electrogenic NaPi-IIa (human isoform), measured under the same conditions, the decrease was typically $\approx 40\%$ (data not shown). To quantify the temperature dependence, the I_{P_i} - V data were transformed and replotted as Arrhenius plots (see Materials and Methods) for test potentials in the range -180 mV to -20 mV (Fig 2B). Data for $V > -20 \text{ mV}$ were too unreliable to fit well and were excluded from analysis. We estimated the activation energy (E_a) from the slopes reported by linear regression fitting (Eqn 3b). This showed little dependence on membrane potential and varied from $43 \pm 2 \text{ kcal mol}^{-1}$ (-180 mV) to $47 \pm 6 \text{ kcal mol}^{-1}$ (0 mV). In contrast, for the human WT NaPi-IIa, E_a at -180 mV was significantly smaller: ($19 \pm 4 \text{ kcal mol}^{-1}$) and increased to $30 \pm 10 \text{ kcal mol}^{-1}$ at -20 mV, where there was more experimental uncertainty in the data (Fig 2C). To establish whether the high E_a for AAD-IIC was caused by the mutations or was an intrinsic property of NaPi-IIC, we performed ^{32}P uptake at different temperatures on WT NaPi-IIC expressing oocytes. For these data, the Arrhenius analysis gave $E_a = 13 \pm 4 \text{ kcal mol}^{-1}$ (*data not shown*).

We next examined the temperature dependence of the presteady-state current relaxations for the AAD-IIC. In the absence of P_i and external interacting cations, voltage steps induce presteady-state currents that reflect charge movements associated with the voltage-dependent reorientation of the unloaded protein alone. In the presence of external Na^+ ions, additional charge movement is detected due to Na^+ interactions (e.g (15)). Fig 3A shows example recordings of membrane currents induced by voltage steps recorded from the same oocyte, superfused with 100Na at two temperatures. By reducing the temperature from 26°C to 22°C, the relaxations slowed, which indicated that the underlying process was temperature dependent. We quantified this behavior by fitting the data with a single exponential decay starting 5 ms after the voltage step. For superfusion with 100Na the ON time constant (τ_{ON}) obtained from fitting relaxations induced by voltage steps from -60 mV to the test potential showed a strong voltage dependence (Fig 3B) that became more marked at lower temperatures. The OFF time constant (τ_{OFF}) was similarly obtained by fitting relaxations for the corresponding return step to -60 mV. As expected, τ_{OFF} was independent of the starting potential and the mean values also increased with decreasing temperature and corresponded approximately with the interpolated value at -60 mV for τ_{ON} (Fig 3B). The increase in τ_{ON} and τ_{OFF} was fully reversible and we confirmed for measurements at 22 °C and 26 °C that the total charge displaced (see Materials and Methods) was unchanged with temperature, as expected (*data not shown*). Resolution of the charge movements at temperatures $< 20 \text{ }^\circ\text{C}$ was difficult because of the compromised signal-to-noise ratio and contamination from endogenous currents that depended on the oocyte batch.

As for the steady-state I_{P_i} analysis, the Arrhenius plots for the relaxation rates (τ_{ON}^{-1} and τ_{OFF}^{-1}) (Fig 3C) showed a linear dependence on $1/T$. For the τ_{ON}^{-1} data, there was a small change in the slopes with V and a vertical shift between plots. In contrast, the plots superimposed for τ_{OFF}^{-1} , consistent with the notion that only the target potential determines the relaxation rate, which in this case, was constant ($= -60 \text{ mV}$). For superfusion with

100Ch, the predicted E_a obtained from linear regression fits to the Arrhenius plots was ≈ 40 kcal mol⁻¹. There was an upward trend with increasing V and the values were comparable with the E_a determined for I_{P_i} . For superfusion with 100Na, the predicted E_a were slightly less than that for I_{P_i} (Fig 2C) and showed a similar upward trend with V .

Presteady-state relaxations at a fixed temperature

The above findings suggested that partial reactions preceding P_i interaction could account for the temperature dependence of AAD-IIc. We next investigated the presteady-state kinetics in more detail at a fixed temperature (≈ 22 - 23 °C) by first focusing on the interaction of Na^+ ions with the empty carrier.

We explored the Na^+ -dependence quantitatively by recording relaxations for equimolar replacement of choline with different $[\text{Na}^+]$. Relaxations recorded from a representative oocyte expressing AAD-IIc were similar whether 100 mM Na^+ was present in the external medium (100Na) or Na^+ was replaced with choline (100Ch) (Fig 4A). To quantify the charge movement, we estimated the charge displacement, by fitting the relaxations with a double exponential function, commencing after completion of the endogenous charging component and integrating the total fit after backward extrapolation (Materials and Methods). We found that this fitting procedure described the relaxations better than using a single exponential fit, as judged by the almost complete elimination of residuals (difference between data and fit) as reported by the fitting algorithm over the fitting window. Moreover, we found that by setting the pre-step holding potential to 0 mV, we were able to resolve the relaxations over the entire test voltage range without significant contamination from endogenous Cl^- currents. These were usually activated for $V > 0$, when stepping from hyperpolarizing potentials and were dependent on the oocyte batch.¹

For all test solutions, the data showed a characteristic sigmoidal shape (Fig 4B) that could be described by a Boltzmann function (Eqn 2). The time constants for the two components (τ_1 and τ_2), plotted as a function of V for different $[\text{Na}^+]$ showed ≈ 5 -fold difference (for $V < 0$ mV) and the slower time constant (τ_2) showed a more obvious voltage dependence with a peak at approximately +30 mV (Fig 4C). There was also little systematic difference in the magnitude or voltage dependence between the respective time constants in 100Ch or 100Na as well as intermediate $[\text{Na}^+]$. This suggested that changing $[\text{Na}^+]$ did not significantly alter the interactions of external Na^+ with AAD-IIc.

The Q - V data were fitted with Eqn 2 to obtain the Boltzmann fit parameters (Q_{max} , z and $V_{0.5}$) (Fig 4B). As expected, changing $[\text{Na}^+]$ did not significantly affect Q_{max} (Fig 4D) and z was similarly insensitive to $[\text{Na}^+]$ (Fig 4E). The mid-point potential ($V_{0.5}$) showed a small depolarizing shift with increasing $[\text{Na}^+]$ of approximately 30 mV for a 10-fold change in $[\text{Na}^+]$ from 10 mM to 100 mM (Fig 4F). For WT human NaPi-IIa and flounder NaPi-IIb, a semi-logarithmic plot of $V_{0.5}$ vs $[\text{Na}^+]$ typically gives a straight line for $[\text{Na}^+] > 10$ mM with a slope of 100-120 mV/10-fold $[\text{Na}^+]$ (2, 33). Compared with the corresponding data for the electrogenic human NaPi-IIa data (33), the AAD-IIc data were shifted vertically and the linear dependence of $V_{0.5}$ on $\log_{10}[\text{Na}^+]$ was not obvious for the range of $[\text{Na}^+]$ used (*inset*, Fig 4F).

We next investigated the effect of adding 1mM P_i to the 100Na buffer. Under these conditions, presteady-state relaxations were still visible (Fig 4A). This differs from the behavior that we have previously

¹ Standard procedures to minimize the contamination by endogenous Ca^{2+} -dependent Cl^- currents, such as preincubation of oocytes in BAPTA-AM, or replacement of external Ca^{2+} with Ba^{2+} did not fully suppress these currents.

1 reported for WT electrogenic SLC34 proteins, whereby 1 mM P_i strongly suppresses presteady-state charge
 2 movements (13, 14, 18). Moreover, the relaxations for hyperpolarizing steps from the holding potential, $V_h = -60$
 3 mV, indicated that more charge movement was induced in the presence of P_i and this charge then returned during
 4 the corresponding OFF step (Fig 4A, *arrowed*). When P_i was added to 100Ch in the superfusion medium the
 5 presteady-state relaxations were unaffected (*data not shown*). This confirmed that the presence of Na^+ was a
 6 prerequisite for P_i interaction with AAD-IIc, consistent with all WT SLC34 isoforms. The results of fitting the
 7 corresponding charge movements with Eqn 2, gave $V_{0.5} = -91 \pm 11$ mV, $z = 0.51 \pm 0.05$ and Q_{max} decreased by 18
 8 $\pm 6\%$ relative to 100Ch ($n = 5$). The main effect of P_i was therefore a hyperpolarizing shift of the equilibrium
 9 distribution of mobile charge. We characterized this by determining the dependence of the shift in $V_{0.5}$ on external
 10 $[P_i]$ (Fig 5). Fig 5A shows pooled, normalised $Q-V$ data ($n = 4$) obtained for the ON transition for different $[P_i]$
 11 indicated. Increasing $[P_i]$ caused a systematic shift of the charge distribution towards hyperpolarizing potentials.
 12 As above, the data were fitted with a single Boltzmann function (Eqn 2). For these data, the uncertainties in the
 13 Boltzmann fit parameters (z , $V_{0.5}$) were reduced by constraining Q_{max} to the value obtained with 0 mM P_i .
 14 Whereas the predicted z remained reasonably insensitive to changes in $[P_i]$ (Fig 5B), the hyperpolarizing shift of
 15 $V_{0.5}$ plotted as a function of $[P_i]$ could be well described by fitting with a modified form of Eqn 1 ($n_H = 1$) (Fig
 16 5C). This yielded a Michaelis constant ($K_{0.5}^{P_i}$) = 1.5 ± 0.5 mM.

25 **Cysteine substitutions in AAD-IIc**

27 Cysteine mutagenesis has proven a useful technique to identify functionally important sites of SLC34
 28 proteins (e.g. (15)). We made Cys-substitutions in AAD-IIc and investigated the constructs by means of
 29 conventional TEVC (steady-state kinetics) and voltage clamp fluorometry (VCF) (7, 19, 20, 34, 35). We chose
 30 sites in AAD-IIc that corresponded to those previously characterised in other SLC34 isoforms (Fig 6, Table 1).
 31 Our rationale was that substitutions at these conserved sites may show similar deviations from WT behaviour as
 32 found for the AAD-IIc kinetics, and thereby allow us to predict structure-function relationships.

36 Before proceeding with the mutagenesis, we first determined if exposure of oocytes expressing AAD-
 37 IIc to methanethiosulfonate (MTS) reagents (MTSET, 1 mM) altered their electrogenic behavior. That no change
 38 was observed confirmed that the triple mutation itself had not exposed any previously inaccessible native
 39 cysteines that might be involved in conformational changes associated with transport function (*data not shown*). All
 40 Cys-mutants gave robust I_{P_i} , with typical magnitudes > 50 nA at -100 mV. With the exception of AAD-IIc-309,
 41 all showed a weak apparent affinity for P_i that is characteristic for AAD-IIc (Table 2). The smaller P_i affinity
 42 constant ($K_{0.5}^{P_i}$) for AAD-IIc-309 indicated that this site may be critically associated with P_i interactions. For the
 43 other constructs, the $K_{0.5}^{P_i}$ values similar to AAD-IIc and indicated that the Cys-substitution had not significantly
 44 affected the substrate interactions. In general, the large variation in $K_{0.5}^{P_i}$ arose from uncertainties in fitting the
 45 weakly saturating dose dependence data with Eqn 1. For 5 of the mutants (AAD-IIc-105, -154, -155, -222, and -
 46 510) the Cys-substitution weakened the voltage dependence of I_{P_i} compared with AAD-IIc alone, whereas for
 47 AAD-IIc-309 and -437 we observed no change in the electrogenic response (Fig 6).

54 The effect on I_{P_i} of exposure of oocytes expressing each mutant to MTSET (1 mM for 5 min) depended
 55 critically on the site of Cys substitution (Fig 1B). Mutants fell into two groups: those in which there was no
 56 change in the magnitude and voltage dependence of I_{P_i} , like AAD-IIc itself, and those that resulted in a marked
 57 alteration (Fig 6, Table 2). We concluded that for the former group the sites of mutagenesis (Ser-155, A-222, and
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D-440) were either not functionally critical in AAD-IIc, or were not accessible from the external medium. The importance of the site is illustrated in the case of mutations in the predicted re-entrant loop (TMD3,4, Fig 1B): modification of the Cys substituted at Val-154 caused a significant hyperpolarizing shift in the voltage dependence of I_{P_i} (Fig 6A, *upper panel*), whereas the construct AAD-IIc-155 with a Cys substituted at the adjacent Ser showed no detectable change in I_{P_i} after incubation (Fig 6A, *lower panel*). Oocytes expressing AAD-IIc with Cys substitution at Ala-222 at one end of the predicted large extracellular loop showed no change in I_{P_i} , whereas at the other end (Leu-309) we found a modest ~25% inhibition in I_{P_i} but with no change voltage dependence (Fig 6B, *lower panel*).

Thiol modification of Cys at sites in the two predicted outer linkers (G105 and L510), showed complementary changes in I_{P_i} (Fig 6C), consistent with previous findings for Cys substituted at the equivalent sites in NaPi-IIa (10). The voltage dependence of AAD-IIc-105 was weak and after modification I_{P_i} increased 4-fold ($V = -100$ mV) to give a voltage dependence that resembled that of AAD-IIc. To verify that this was a direct result of the modification of Cys-105, we exposed oocytes expressing AAD-IIc-105 and pre-labeled with MTSEA to the reducing agent DTT. This resulted in a reversion of the electrogenic response close to the original $I-V$ relation (Fig 6C, *upper panel*). In contrast, AAD-IIc-510 showed the opposite behaviour, whereby I_{P_i} was inhibited by ~50% and after thiol modification, its voltage dependence was similar to AAD-IIc-105 before MTS exposure (Fig 6C, *lower panel*).

Finally, two mutants involving Cys substitutions at nearby locations in the predicted linker between TMD9 & 10 also displayed contrasting behavior. Whereas mutant AAD-IIc-440 showed no resolvable change after incubation (*data not shown*), exposure of oocytes that expressed AAD-IIc-437 to MTS reagents resulted in a significant suppression of I_{P_i} (Fig 6D, *upper panel*). For this construct, the typical $I-V$ response to 1 mM P_i reversed at -30 mV and displayed a negative slope. This behavior has been previously interpreted to represent full inhibition of cotransport function and recapitulates the behavior of other electrogenic SLC34 isoforms with a Cys at the equivalent site (Table 1, see Discussion) (25, 35). To quantitate the Cys-modification reaction for AAD-IIc-437 and investigate if membrane potential could influence the accessibility of the Cys, we applied MTSET (10 μ M) for 1 min intervals at a defined holding potential and measured I_{P_i} at -50 mV after each exposure. The data were fitted with a single decaying exponential function (Eqn 4) to yield the effective pseudo first order rate constants (see Materials and Methods): $1.57 \pm 0.1 \text{ mM}^{-1} \text{ s}^{-1}$ (0 mV); $1.18 \pm 0.03 \text{ mM}^{-1} \text{ s}^{-1}$ (-50 mV); $1.63 \pm 0.05 \text{ mM}^{-1} \text{ s}^{-1}$ (-90 mV) ($n = 4$) (Fig 6D, *lower panel*).

Labeling with MTS-TAMRA and changes in emitted fluorescence intensity

For voltage clamp fluorometry (VCF) measurements, we incubated oocytes expressing each of the Cys-mutants in a medium containing the fluorophore MTS-TAMRA and tested them for voltage-induced changes in emitted fluorescence intensity under different substrate superfusion conditions (100Na, 100Ch and 100Na+1 P_i). Three of the constructs (AAD-IIc-154C, AAD-IIc-222 and AAD-IIc-437) gave measurable ΔF and were characterised in detail.

AAD-IIc-437: After labelling with MTS-TAMRA, oocytes expressing AAD-II-437 showed the expected suppressed electrogenic response to 1 mM P_i , consistent with the behavior of this mutant when labelled with MTSET (Fig 6D). We first examined if the steady-state fluorescence was dependent on the superfusate, as we reported for the labelled mutant S437C in NaPi-IIc (20). Indeed oocytes expressing AAD-IIc-437 and voltage clamped at -60 mV showed up to 3% reversible quench of F (*data not shown*), when switching the superfusate

from 100Ch to 100Na. All other experiments were performed using a voltage step protocol (see Materials and Methods). Representative voltage-induced ΔF recordings are shown in Fig. 7A. For $V < 0$, little or no ΔF was detected whereas for strongly depolarizing signals ΔF was quenched in a voltage- and substrate-dependent manner. The quenching was the strongest in the absence of external Na^+ (Fig 7B) and at a given V , addition of Na^+ to the external medium reduced the amount of quenching in a dose-dependent manner. Saturation of the ΔF - V data was not observed at the depolarizing extreme and we were unable to reliably voltage clamp oocytes for $V > 200\text{mV}$. In the presence of 100 mM Na^+ , addition of P_i to the external medium reduced the amount of quenching further in a dose-dependent manner (Fig 7A, C).

AAD-IIC-154: based on a previous study on the flounder NaPi-IIb isoform (34) we had expected that labelling a Cys substituted at the equivalent site (S155) in AAD-IIC would result in voltage induced ΔF . Surprisingly, we could not resolve ΔF from oocytes expressing the construct AAD-IIC-155 (data not shown). However, Cys-substitution at the neighbouring site (V154) gave a functional construct that exhibited robust ΔF (Fig 8A). The voltage step induced ΔF was similar for superfusion in 100Ch and 100Na, however the addition of P_i suppressed ΔF for depolarizing steps and increased ΔF for hyperpolarizing steps. The ΔF - V data were fit with a single Boltzmann function (Eqn 2, see Materials and Methods) to yield estimates for the midpoint voltage ($V_{0.5}^F$) and slope factor (z^F). Unconstrained fits confirmed that F_{max} was largely insensitive to manipulations of the external $[\text{Na}^+]$ (Fig 8B, *upper*). The predicted $V_{0.5}^F$ showed a modest increase from $+51 \pm 9$ mV (100Ch) to $+71 \pm 11$ mV (100Na) whereas z^F remained unchanged (0.39 ± 0.06 (100Na) and 0.38 ± 0.05 (100Ch)). We then quantitated the effect of progressively increasing external $[\text{P}_i]$ (in 100Na) (Fig 8B, *centre*). This resulted in a systematic hyperpolarizing shift in $V_{0.5}^F$, (Fig 8B *lower*) together with a reduction in the slope factor z^F from 0.43 ± 0.02 (0 mM P_i) to 0.29 ± 0.01 (2 mM P_i) ($n = 4$). The dependence of $V_{0.5}^F$ on $[\text{P}_i]$ could be described analytically with a modified form of the Michaelis function (Eqn 1 ($n_H=1$)) to give an apparent $K_{0.5}^{\text{P}_i} = 1.04 \pm 0.7$ mM (Fig 8B, *lower*).

AAD-IIC-222: Oocytes expressing this mutant showed no significant change in electrogenic activity after labelling (Fig 6B, *upper*), yet robust ΔF - V was resolved. Like AAD-IIC-154, there was little change in ΔF when replacing choline with Na^+ in the superfusate, however when P_i (1 or 2 mM) was added to the superfusate (100Na), ΔF increased for hyperpolarizing voltage steps and decreased for depolarizing steps relative to the holding potential, like AAD-IIC-154. The ΔF - V data with variable $[\text{Na}^+]$ were fit with the Boltzmann function (Eqn 2) (Fig 8C, *upper*) and the Boltzmann analysis revealed a small depolarizing shift $V_{0.5}^F$ as $[\text{Na}^+]$ increased: with 100Ch, $V_{0.5}^F = -2.2 \pm 5.2$ mV and at 100Na, $V_{0.5}^F = +16.0 \pm 4.9$ mV. In contrast, F_{max} was relatively insensitive to this manipulation and z^F decreased from 0.57 ± 0.06 (100Ch) to 0.48 ± 0.05 (100Na). We also investigated the effect of adding P_i to the 100Na medium (Fig 8C, *centre*). This caused a hyperpolarizing shift of $V_{0.5}^F$, without significantly affecting F_{max} . For this mutant, $V_{0.5}^F$ also showed evidence of saturation at high $[\text{P}_i]$ and the dependence of $V_{0.5}^F$ on $[\text{P}_i]$ could be described analytically in terms of a modified form of the Michaelis fit (Eqn 1, $n_H=1$) to give $K_{0.5}^{\text{P}_i} = 0.22 \pm 0.1$ mM (Fig 8C, *lower*).

Discussion

For many Na⁺-coupled solute carriers, transmembrane electrical potential is a source of free energy to drive coupled transport, in addition to the chemical energy imparted by the Na⁺ concentration gradient. The effect of membrane potential is manifested as the voltage dependence of transport rates (substrate-induced currents) assayed under voltage clamp conditions. Hyperpolarizing membrane potentials increase the inwardly directed transport-associated membrane current and this current correlates directly with solute flux (e.g. (2, 17, 27-29)). Voltage dependence of transport rate implies that there must be one or more partial reactions in the transport cycle whose rate constants are membrane potential dependent. Furthermore, these partial reactions must involve charge displacements that could be either intrinsic to the protein, or arise from the driving cations moving within transmembrane electric field. Such charge displacements are thought to accompany protein conformational changes. It is also possible that the overall transport cycle is electroneutral and one or more partial reactions are voltage dependent and become rate limiting (26), although this not been demonstrated experimentally in the case of SLC34.

For AAD-IIC, which displays 3 hallmarks of electrogenic SLC34 cotransport (net charge translocation, 3:1 Na⁺:HPO₄²⁻ stoichiometry and presteady-state charge displacement), we have proposed that its electrogenicity is a direct result of the re-establishment of the cotranslocation of the Na⁺ ion associated with transition 1↔2 during the cotransport cycle, as proposed for the WT electrogenic isoforms (4). Thus, according to our kinetic scheme, (Fig 1A), the salient difference between the electrogenic and electroneutral SLC34 proteins concerns partial reactions between states 0, 1, 2 and 9. This is also supported by the presence of a leak mode for electrogenic SLC34 proteins, that we have hypothesised to involve the translocation of a single Na⁺ ion per cycle, in the absence of P_i and which can be described kinetically by transitions between these 4 states (1)². In this study, we used AAD-IIC together with mutant constructs involving Cys-substitutions at sites previously identified in studies on the electrogenic (NaPi-IIa/b) and electroneutral (NaPi-IIC) to gain new insights into the mechanism of electrogenic cotransport.

Electrogenicity of the empty carrier imposes an energetic cost to AAD-IIC

Although AAD-IIC displays the qualitative features expected for an electrogenic SLC34 protein, its kinetic characteristics depart significantly from that of the WT electrogenic isoforms, which implied that the mutagenesis altered the kinetics of one or more partial reactions in the cotransport cycle. To identify these reactions, we first examined the temperature dependence of the kinetics of cotransport and cation interactions. Solute coupled carrier proteins generally exhibit a high temperature dependence that differs significantly from that of ion channels and can be used to distinguish between diffusional processes and those involving conformational changes (3, 5, 6, 21). With respect to steady-state cotransport rate (I_p), we estimated that E_a for the electrogenic human NaPi-IIa and AAD-IIC was respectively ≈2- and ≈3-fold larger than for the corresponding P_i uptake rate of the electroneutral NaPi-IIC (Fig 2C). This was clear evidence that cotransporting an additional Na⁺ ion by the electrogenic isoforms invoked a larger energetic cost. Moreover, our finding that E_a for AAD-IIC was significantly greater than for NaPi-IIa indicated that the mutagenesis in NaPi-IIC had resulted in a less efficient transporter. Given that the cotransport cycle involves a number of partial reactions, one or

² We also previously reported evidence of a leak mode for AAD-IIC based on the electrogenic response to application of the blocker phosphonoformic acid (PFA) (4). A detailed characterisation of this mode was not undertaken in the present study due to the low apparent affinity of AAD-IIC for P_i and PFA that would result in incomplete suppression of the leak current with the substrate concentrations used.

more of which might be responsible for the overall high E_a , we limited the number of possible states the proteins could populate and focussed on the temperature dependence of presteady-state relaxations in the absence of P_i . In the absence of external Na^+ (100Ch) E_a was essentially unchanged (Fig 3D), we therefore propose that the high E_a of AAD-IIc results from partial reactions that involve neither external Na^+ ions nor P_i , with the empty carrier ($0 \leftrightarrow 1$, Fig 9A) being the most likely candidate. We cannot explicitly discount the contribution of partial reactions between “inward facing” states, however with low activity of substrates in the cytosol, the probability of occupancy of these states is expected to be concomitantly low. It is therefore most likely that the introduction of the charged Asp-196 into NaPi-IIc has resulted in an electrogenic transition that represents a conformational change between states 0 and 1. This transition is energetically unfavourable compared to the same transition in the WT electrogenic isoforms (3) and would be expected to involve a concomitantly larger conformational change.

A 7-state model accounts for the kinetic properties of AAD-IIc

We obtained further insight into the AAD-IIc kinetics, by performing numerical simulations using a set of differential equations that described the rate of change of state occupancy of a reduced form of the 10-state scheme (Fig 9A). This 7-state scheme allowed us to investigate the steady-state and presteady-state behavior of AAD-IIc with a workable number of free parameters and compare its behaviour with corresponding experimental data. We lumped the interactions of P_i and the final Na^+ ion before translocation together, but took account of two Na^+ ions interacting sequentially before P_i binding and we further simplified the ordered sequence of substrate interactions at the cytoplasmic face by assuming that rapid equilibrium conditions applied to the transitions between states 7 and 9 (Fig 1A). The simulations successfully predicted the main features of the presteady-state current relaxations (Fig 9B), namely the weak influence of external Na^+ on charge movement and the presence of a significant charge movement in the presence of external P_i . For comparison, selected traces from representative data (Fig 4A) are also shown.

Based on the presteady-state data obtained for AAD-IIc in the absence of external Na^+ (100Ch), the effective charge displacement of the empty carrier was the same as in the original model ($0.4 e^-$), however the assignment of voltage dependence to the partial reactions $1 \leftrightarrow 2$ and $9 \leftrightarrow 0$ differed in two respects. First, it was necessary to assign a weaker interaction of the first Na^+ ion ($1 \leftrightarrow 2$) with the transmembrane electric field (effective valence = $0.1 e^-$) to account for: (i) the absence of a strong effect of external $[Na^+]$ on the presteady-state charge distribution and the total detectable charge displacement (Fig 4B; 9C) and (ii) the weak hyperpolarizing shift in the midpoint voltage ($V_{0.5}$) of the Q - V distribution (Fig 4F; 9C, *lower panel*). Second, we increased the effective valence of the partial reaction that accounts for the release of the 3rd Na^+ ion on the cytosolic side ($9 \leftrightarrow 0$) to $0.5 e^-$. Together with a slow backward rate (Na^+ release; $k_{09} = 7 s^{-1}$), and a highly asymmetrical barrier for the transition $0 \leftrightarrow 9$, we could also account for: (i) the increased charge movement for hyperpolarizing steps in the presence of P_i (Fig 4A; 9B, *right panel*) and (ii) the hyperpolarizing shift in $V_{0.5}$ with increasing $[P_i]$ (Fig 5C; 9D). Moreover, this showed Michaelian behavior (Fig 9D, *lower panel*) in accord with the analysis of the experimental data (Fig 5C). In the absence of external P_i and the low internal $[Na^+]$, the state occupancy is distributed between states 0, 1 and 2. When external P_i is present, state 9 can accumulate after ordered release of 2 Na^+ ions and P_i to the cytosol ($6 \rightarrow 9$). The completion of the cotransport cycle only occurs after release of the 3rd Na^+ ion, which gives rise to the presteady-state charge movement induced by hyperpolarizing voltage steps (Fig 9B, *right panel*) and to a net inward cotransport current in the steady-state.

The most important functional characteristic of AAD-IIC that differed from the WT transporters is the apparent affinity constant for P_i ($K_{0.5}^{P_i}$) that we originally reported to be >100 fold higher than that of either the electrogenic or electroneutral isoforms (4). This suggested that the triple mutation of NaPi-IIC had specifically affected P_i interactions with the protein. In general, although $K_{0.5}^{P_i}$ can be influenced by all rate constants (forward and backward) associated with partial reactions of the transport cycle, likely candidates could also include those associated with Na^+ ion interactions that precede external P_i binding. As expected, our simulations confirmed that $K_{0.5}^{P_i}$ was dependent on the ratio of forward to backward rate constants associated with transitions $1 \leftrightarrow 2$, $2 \leftrightarrow 3$ and $3 \leftrightarrow 5$. However, given the constraints on the model parameters imposed by having good agreement with all the experimental data, we found that partial reaction $3 \leftrightarrow 5$ was indeed the most critical determinant of $K_{0.5}^{P_i}$. Specifically, the ratio k_{12}^0/k_{21}^0 was constrained by the dependence of $V_{0.5}$ on external $[Na^+]$ (2) and was fixed ≈ 5 in the simulations to give reasonable agreement with measurement. Furthermore, although altering k_{23}^0/k_{32}^0 had little influence on the simulated presteady-state behaviour in 0 mM P_i , this ratio was also found to be a critical determinant of the apparent affinity constant for Na^+ at a given $[P_i]$, ($K_{0.5}^{Na}$), and the cooperativity of Na-activation of I_{P_i} (4). Thus, for AAD-IIC, the low apparent affinity for P_i compared to the electrogenic and electroneutral isoforms most likely results from the effect of the mutagenesis on the P_i binding partial reaction ($3 \leftrightarrow 6$) and is largely unaffected by the preceding Na^+ interactions. The final set of parameters that gave good agreement with the steady-state data (4) were consistent with a cooperative interaction of two Na^+ ions preceding P_i binding ($k_{12}^0/k_{21}^0 < k_{23}^0/k_{32}^0$) and with k_{35}^0/k_{53}^0 adjusted to give $K_{0.5}^{P_i} \approx 1$ mM and $K_{0.5}^{Na} \approx 110$ mM (at -60 mV) (Fig 9E), similar to previously reported values (4).

In summary, the simulations helped to identify two critical partial reactions that contribute to the AAD-IIC kinetics: (i) the cytosolic release of one Na^+ ion from the protein that establishes a rate limiting step in the transport cycle to define the maximum transport rate, and (ii) the external P_i interaction which defines the apparent affinity for P_i .

Fluorometric assays complement presteady-state relaxation analysis

The altered kinetic behavior of AAD-IIC compared to the WT proteins indicated that mutagenesis had modified the voltage- and substrate-dependence of the conformational changes during the transport cycle. To relate these kinetic findings to dynamic changes in the molecular structure, we substituted Cys at sites that previous studies had suggested were involved in unique conformational changes determined by membrane potential and substrate availability (10, 11, 20, 23, 25, 34). Cys substitution itself was well-tolerated and similar phenotypes were observed after thiol modification, exemplified by mutants AAD-IIC-437, AAD-IIC-105 and AAD-IIC-510.

Exposure of oocytes expressing AAD-IIC-437 to MTS reagents resulted in suppression of cotransport activity, as we have previously reported for the equivalent mutation in other SLC34 isoforms (20, 24, 25, 35) (Fig 6D). Moreover, the pseudo 1st order rate constant for the Cys-modification reaction was similar to values we previously reported for modification of a Cys at the equivalent site in rat NaPi-IIa (S460C) (25) and the flounder NaPi-IIb (S448C) (35). This indicated that external accessibility of this critical site was not changed and moreover, the accessibility was also insensitive to membrane potential ($V \leq 0$ mV). The voltage dependence of mutants AAD-IIC-105 and AAD-IIC-510 (Fig 6C) also showed qualitatively similar behavior as previously reported for the equivalent Cys-substitutions in the rat NaPi-IIa (11) whereby Cys-modification resulted in a complementary change in the voltage dependence. These findings not only confirmed that AAD-IIC has a similar

topology to NaPi-IIa at these critical sites, but that the functional consequences of Cys-modification at these sites was comparable.

The substituted Cys were labelled with fluorophores to act as reporters of local conformational changes (19, 20, 34, 35). The behavior of labelled oocytes expressing AAD-IIc-437 in response to changing the superfusate from 100Na to 100Ch was qualitatively the same as we previously reported for the NaPi-IIc mutant S437C (20): in the steady-state ($V_h = -60$ mV), addition of Na^+ quenched the fluorescence. However, the response of AAD-IIc-437 to voltage steps differed from the electrogenic NaPi-IIb mutant S448C (35), whereby under all superfusion conditions there was resolvable voltage-induced change in fluorescence intensity (ΔF) *only* for depolarizing voltage steps (Fig 7). Superfusion with 100Ch gave the largest quench of ΔF at a given positive test potential and this diminished in a concentration-dependent manner with the addition of more external Na^+ as well as with the addition of external P_i . In contrast, for the electrogenic S448C mutant in flounder NaPi-IIb (35), no resolvable ΔF was reported for voltage steps from -60 mV to test potentials >0 mV, and increasing external $[\text{Na}^+]$ quenched ΔF for hyperpolarizing potentials. Similarly, for S437C mutant made in the mouse NaPi-IIc we reported that the addition of external Na^+ caused a quenching of the steady-state fluorescence (20) as we also observed for the steady-state F reported by AAD-IIc-437.

One interpretation of the behavior of AAD-IIc-437 is that the microenvironment of the fluorophore at site 437 reports Na^+ interactions from *either* side of the membrane, depending on the membrane potential and availability of Na^+ ions. This would be consistent with the alternating access mechanism for cation driven transport (e.g. (12)). Thus, for strongly depolarizing voltage steps, and *no* external Na^+ , cytosolic Na^+ ions would be driven to their binding site (partial reaction $0 \rightarrow 9$), thus giving rise to ΔF when superfusing with external 100Ch (Fig 6B). As fluorescent quenching is thought to result from exposure of the fluorophore to a hydrophilic environment (e.g. (8)), this behavior would suggest that this site becomes more exposed to the cytosol as *internal* Na^+ ions bind to the protein. By increasing *external* $[\text{Na}^+]$, the state distribution of AAD-IIc would be altered to favour states 1 and 2 (external binding of Na^+) with little voltage-dependent conformational change when external Na^+ binds, consistent with our presteady-state relaxation findings. Under these conditions, site 437 would become exposed to the external medium and thereby account for the quenching of steady-state fluorescence in the presence of external Na^+ . The addition of external P_i (in the presence of external Na^+) would further diminish the effect of the cytosolic Na^+ interaction on ΔF , as the transport cycle proceeds in the forward (clockwise direction, Fig 9A). That we did not observe ΔF for depolarizing voltage steps with the S448C flounder NaPi-IIb mutant (35) suggests that for the WT electrogenic isoforms, the conformational changes associated with the binding and release of the last Na^+ ion to the cytosol ($9 \leftrightarrow 0$) are small compared to AAD-IIc. This is also supported by the model prediction, whereby this Na^+ ion would move through a smaller fraction of the transmembrane electric field (Fig 9A).

The other two AAD-IIc Cys mutants from which we could detect fluorescence changes (AAD-IIc-154, AAD-IIc-222) showed behavior consistent with the altered kinetics of AAD-IIc. The ΔF reported by a fluorophore linked to the Cys -154 (AAD-IIc-154) can be compared with that for the equivalent mutant in flounder NaPi-IIb (S155C) (34). This mutant shows strong dependence of ΔF on external $[\text{Na}^+]$ (slope of $V_{0.5}$ vs $\log [\text{Na}^+] = 140$ mV/decade), unlike the weak dependence we report here for AAD-IIc-154 (Fig 8A,B). Moreover, for S155C when external $[\text{P}_i]$ is changed in the presence of 100 mM Na^+ , we observe strong quenching, but no obvious shift in $V_{0.5}^F$, whereas AAD-IIc-154 showed a hyperpolarizing shift (Fig 8B) that was qualitatively similar to the shift found for presteady-state relaxations with variable $[\text{P}_i]$ (Fig 5C) and comparable

to the transport $K_{0.5}^{P_i}$ (Table 1). The insensitivity to changes in external $[Na^+]$ for AAD-IIC-154 indeed suggested that the microenvironment of the fluorophore at Cys -154 reports similar conformational changes to those that give rise to the presteady-state charge movement: reorientation of the empty carrier, including the release of the final Na^+ ion to the cytosol during cotransport. Qualitatively similar behaviour was found for the $\Delta F-V$ data obtained by labelling mutant AAD-IIC-222. Taken together, these findings suggest that the local environments of these sites undergo voltage-dependent conformational changes associated with the empty carrier and cytosolic Na^+ release.

Conclusions

Electrogenicity can be conferred to the electroneutral Na^+ -coupled P_i cotransporter (NaPi-IIC) by the substitution of 3 amino acids, one of which is negatively charged (D195). We hypothesize that the movement of this charge (evidenced by presteady-state relaxations), results in a conformational change of the empty carrier between inward and outward facing orientations (evidenced by fluorometric recordings), that allows a single Na^+ ion to bind from either side of the membrane, subsequently translocate and dissociate, driven by the electrochemical gradient for Na^+ . Whether or not this charge itself is part of the cation coordination or indirectly exposes a binding site cannot be determined without 3-D structural information. Certainly, for this charged residue to fulfil the role as the empty carrier “voltage sensor”, the current secondary topology model of SLC34 proteins (15), which places D195 close to the cytosolic end of TMD5 and effectively outside the transmembrane electric field (Fig 1B), would require reassessment.

Although AAD-IIC can be considered an “imperfect” transporter, with a significantly greater E_a than the WT electroneutral and electrogenic isoforms, its deviation from WT behavior has revealed an important partial reaction in the physiologically relevant transport cycle, namely the cytosolic release of a Na^+ ion prior to reorientation of the empty carrier. For AAD-IIC, the kinetics of this transition impose a rate-limiting step on the transport cycle, consistent with this ion being more tightly bound to AAD-IIC than for the electrogenic WT isoforms. Identification of this partial reaction in the electrogenic WT would otherwise be difficult to achieve experimentally using the intact oocyte system. The cytosolic dissociation of this Na^+ ion, revealed through mutagenesis, means that AAD-IIC provides a unique tool to investigate cation interactions at the internal protein interface for SLC34 proteins.

We originally identified 3 critical regions in the SLC34 sequences that differed significantly between electrogenic and electroneutral isoforms (4). Although one of these regions contains D195 (or its equivalent) found in the electrogenic isoforms, the roles played by other dissimilar polar and non-polar residues in the other regions in establishing a voltage dependent interaction of external Na^+ ions, together with the high apparent affinity for P_i , remain to be elucidated.

Table 1
Comparison of selected Cys-substitution sites among SLC34 isoforms (see Fig 6)

Protein	Site of Cys-substitution							
mAAD-IIc	G105	V154	S155	A222	L309	S437	D440	L510
rNaPi-IIa	G134 (10, 11)		S183 (30)			S460 (24, 25)	E463 (25)	M533 (10, 11)
fNaPi-IIb		S155 (19, 35)		S226 (34)	Q319 (34)	S448 (19, 35)		
mNaPi-IIc						S437 (20)		

Blank entry indicates constructs that have not been synthesised or investigated in our laboratory. Equivalent sites indicated according to a multiple sequence alignment for the isoforms and species indicated, where f= flounder, m= mouse, r= rat.

Relevant citations given in parentheses.

Table 2
Summary of properties of Cys mutations in AAD-IIc (see Fig 6)

Property	AAD-IIc	+G105C	+V154C	+S155C	+A222C	+L309C	+S437C	+D440C	L510C
$K_{0.5}^{P_i}$ -60 mV (mM)	6.5±4.8 (4)	n.d.	1.1±0.4	2.9±0.8	1.5±0.4	0.6±0.1	1.6±0.4	2.0±.8	1.5±0.3
$\frac{I_{P_i}^{+MTS}}{I_{P_i}^{-MTS}}$	↔	↑	↓	↔	↔	↓	↓	↔	↓
ΔF	No	No	Yes	No	Yes	No	Yes	No	No

Symbols: ↔: indicates that ratio $I_{P_i}^{+MTS}/I_{P_i}^{-MTS}$ remains unchanged; ↑ indicates that ratio $I_{P_i}^{+MTS}/I_{P_i}^{-MTS}$ increased; ↓ indicates that ratio $I_{P_i}^{+MTS}/I_{P_i}^{-MTS}$ decreased. n.d.: not determined.

Acknowledgements

We gratefully acknowledge Eva Hänsenberger for oocyte preparation. Special thanks to Dr Anne-Kristine Meinild (UZH) and Dr Donald D.F. Loo (UCLA) for their insightful comments. This work was supported by the Swiss National Science Foundation grant to ICF.

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Figure legends

Figure 1 SLC34 protein structure-function background

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3 A) A 10-state kinetic scheme for SLC34 proteins describes the electrogenic transport cycle. Shaded states and
4 associated transitions are postulated to be occur in the electroneutral NaPi-IIc transport cycle (see (20)). Boxed
5 values indicate effective valence associated with 3 partial reactions for the electrogenic NaPi-IIa/b. Transition
6 (2 \leftrightarrow 9) accounts for uncoupled Na⁺-leak (see (1)). The empty carrier is assumed to occupy one of two states
7 (“inward” or “outward” oriented), transitions between which involve the movement of an equivalent 0.4 e⁻ across
8 the transmembrane electric field. Hyperpolarizing potentials increase the probability of occupancy of state 1,
9 which allows the sequential binding of two Na⁺ ions to their respective binding sites within the transmembrane
10 electric field and is equivalent to 0.4 e⁻ moving across the transmembrane electric field. This is followed by
11 binding of HPO₄²⁻ and a 3rd Na⁺ to form an electroneutral complex followed by substrate translocation. Substrate
12 release to the cytosol is also assumed to be ordered. The final release of the 3rd Na⁺ ion is modelled as a weakly
13 voltage dependent (0.2 e⁻ equivalent charge) transition that leaves the protein in an “inward” oriented empty
14 carrier state (state 0) and a reorientation to state 1 occurs under the influence of the transmembrane electric field.
15 B) Secondary topology model showing the 3 sites in NaPi-IIc critical for restoring electrogenicity to form the
16 AAD-IIc construct (*triangles*). For Cys-mutagenesis of AAD-IIc, cysteines were substituted individually at sites
17 predicted to be externally accessible (*circles*). Sites that were labelled with a fluorophore, as evidenced by
18 resolvable voltage-dependent changes in fluorescence, are indicated (*filled circles*). Sites were selected to
19 correspond to previous data obtained from Cys mutagenesis studies of SLC34 isoforms (see Table 1). Inset
20 shows the sequence alignment for the indicated region to emphasize the high degree of identity in the predicted
21 transmembrane domain regions and the lack of charge at site 195 for the electroneutral NaPi-IIc. Abbreviations
22 for species prefixes: ,f: flounder, h: human, m: mouse, r: rat, z: zebra fish. Numbering according to mouse NaPi-
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Figure 2. Temperature dependence of electrogenic cotransport mediated by AAD-IIc

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37 A) Current-voltage relationship of P_i-induced current (I_{P_i}) at different temperatures indicated. Data were
38 normalised to I_{P_i} measured at 24 °C and -100 mV. Data pooled from 8 oocytes from 2 donor animals. Points
39 joined by lines for visualisation.
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41 B) Arrhenius plot of AAD-IIc I_{P_i} -V data from A) transformed (see Materials and Methods) at selected potentials.
42 Straight lines generated by linear regression analysis.
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44 C) Activation energy (E_a) obtained from slope of linear regression fits to Arrhenius plot data for AAD-IIc (filled
45 squares) and human NaPi-IIa (hIIa) (open squares) plotted as a function of membrane potential. Dotted line
46 indicates E_a determined for the electroneutral NaPi-IIc (mouse isoform).
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Figure 3. Temperature dependence of AAD-IIc presteady-state relaxations

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53 A) Representative current relaxations recorded from an oocyte expressing AAD-IIc at 26 °C and 22 °C ,
54 superfused in 100Na. Voltage steps shown are from the holding potential of -60 mV to potentials in the range -
55 180 mV to +80 mV.
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57 B) Relaxation time constant for ON (τ_{ON} , *filled symbols*) and OFF (τ_{OFF} , *open symbols*) steps measured as a
58 function of the test membrane potential for the temperatures indicated ($n=4$). For the OFF relaxations, the data
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points indicate the mean \pm sem of τ_{OFF} determined from the pooled data at each step. Data points joined for visualisation.

C) Arrhenius plots for presteady-state relaxation rate (τ_{ON}^{-1} and τ_{OFF}^{-1}) for superfusion with 100Ch and 100Na for steps to selected test potentials. Data shown for ON step (from -60 mV holding potential to test potential indicated) (*left panels*) and OFF step (from test potential to holding potential) (*right panels*). Straight lines are linear regression fits. Data pooled from 4 oocytes.

D) Activation energies (E_a) for the presteady-state relaxation time constants, obtained from slope of linear regression fits to Arrhenius plot data for ON steps (*filled squares*) OFF steps (*open squares*) plotted as a function of membrane potential for the superfusion condition indicated.

Figure 4. Dependence of AAD-IIc presteady-state relaxations on external $[\text{Na}^+]$.

A) Presteady-state relaxations recorded in response to voltage steps from a voltage potential (V_h) = -60 mV to test potentials in the range -160 to +60 mV for 3 superfusion conditions indicated. Dotted lines indicate zero holding current level. For superfusion with 100Na+1P_i, the presteady-state relaxations were altered such that more charge movement was induced for steps to and from hyperpolarizing potentials (*arrows*).

B) Typical Q - V data from a representative oocyte superfused with different $[\text{Na}^+]$ as indicated, for steps from two holding potentials ($V_h=0, -60$ mV). Data are shown for the ON steps. Continuous lines are fits with Eqn 2.

C) Voltage dependence of the two fitted time constants (τ_1, τ_2) for the same superfusion conditions as in B. Data pooled from 4 oocytes. Lines join points corresponding to superfusion with 100Ch and 100Na.

D) Total charge (Q_{max}) displaced obtained from Boltzmann fits to data from individual oocytes using Eqn 2 plotted a function of $[\text{Na}^+]$. Data were normalized to Q_{max} from 100Ch data set. Data pooled from 5 cells.

E) Effective valence (z) obtained from Boltzmann fits to data from individual oocytes using Eqn 2 plotted as a function of $[\text{Na}^+]$. Data pooled from 5 cells. Open squares are data at 0 mM and 100 mM Na^+ for the human NaPi-IIa from (33).

F) Midpoint voltage ($V_{0.5}$) from Boltzmann fits to data from individual oocytes using Eqn 2 plotted as a function of $[\text{Na}^+]$ obtained (*left panel*). Data were replotted on a $\log_{10}[\text{Na}^+]$ scale (*inset, filled symbols*). Equivalent data for the human NaPi-IIa are shown (*open symbols*) for comparison (data replotted from (33)).

Figure 5. Dependence of AAD-IIc presteady-state relaxations on external $[\text{P}_i]$.

A) Typical Q - V data from a representative oocyte superfused with different $[\text{P}_i]$ as indicated, for steps from two holding potentials ($V_h=0, -60$ mV). Data are shown for the ON steps. Continuous lines are fits with Eqn 2.

B) Effective valence (z) obtained from Boltzmann fits to data from individual oocytes, using a modified form of the Eqn 1 ($n_H=1$) plotted as a function of $[\text{P}_i]$. Data pooled from 4 cells.

C) Midpoint voltage ($V_{0.5}$) from Boltzmann fits to data from individual oocytes using a modified form of Eqn 1 ($n_H=1$) plotted as a function of $[\text{P}_i]$ obtained.

Figure 6. Cysteine mutagenesis in the AAD-IIc constructs-dependence of P_i -dependent current (I_{P_i}) on voltage before and after MTS incubation

After determination of the control I_{P_i} vs V relationship (*filled squares*), oocytes were incubated in 1 mM MTSET for 5 min (*open squares*) and the assay repeated. Data shown normalised to I_{P_i} at -100 mV control condition.

Mean \pm sem for ≥ 3 oocytes. Error bars smaller than symbol size not shown. Normalised data ($n=4$) for the AAD-IIC are superimposed on each plot for comparison (*grey filled circles*).

A) For two mutants with adjacent Cys-substitutions in the linker between predicted TMD-4 and TMD-5 (Fig 1B) AAD-IIC-154 (*upper*) showed a hyperpolarizing shift in the voltage dependence of I_{P_i} , whereas AAD-IIC-155 (*lower*) showed no change after MTS incubation.

B) I_{P_i} vs V data for two mutants with Cys-substitutions at each end of the large extracellular loop (Fig 1B), AAD-IIC-222 (*upper*) and AAD-IIC-309 (*lower*). Only AAD-IIC-309 showed a significant change after MTS incubation.

C) I_{P_i} vs V data for Cys-mutants AAD-IIC-105 (*upper*) and AAD-IIC-510 (*lower*) that showed reciprocal changes of I_{P_i} after MTS incubation. After MTS modification, one representative AAD-IIC-105 expressing oocyte was exposed to DTT (10 mM, 30 min), which caused a reversion to the initial response (*filled circles*).

D) *Upper*: Mutant AAD-IIC-437 showed complete suppression of cotransport behaviour after MTS incubation with an outward I_{P_i} for hyperpolarizing potentials. *Lower*: Loss of cotransport activity for mutant AAD-IIC-437 following modification of Cys-437. Normalized response plotted as a function of cumulative exposure time for MTSET (10 μ M) exposure with the individual oocytes voltage clamped during incubation to the potential indicated. Continuous lines are fits with Eqn 4. See text for more details.

Figure 7. Voltage clamp fluorometry (VCF) recordings from oocytes expressing AAD-IIC-437 and labelled with the fluorophore MTS-TAMRA.

A) Representative VCF recordings from the same oocyte expressing AAD-IIC-437 for the voltage step protocol shown and for the different superfusion conditions indicated. Data traces offset-adjusted to the steady-state fluorescence level at $V_h = -60$ mV.

B) Change in fluorescence (ΔF) plotted as a function of V for oocytes expressing AAD-IIC-437 with external $[Na^+]$ varied as indicated. Data for individual oocytes were corrected from photobleaching and loss of fluorescence during the experiment (see Materials and Methods) and pooled ($n=5$) by first normalizing to F_{max} predicted from a fit to the 100Ch data using Eqn 2.

C) Change in fluorescence (ΔF) plotted as a function of V oocytes expressing AAD-IIC-437 with external $[P_i]$ varied as indicated in the presence of 100 mM Na^+ . Data from individual oocytes were corrected for photobleaching and loss of fluorescence during the experiment (see Materials and Methods) and pooled ($n = 5$) by first normalizing to F_{max} predicted from a fit to the 100Ch, 0 mM P_i data using Eqn 2.

Figure 8. Voltage clamp fluorometry (VCF) recordings from oocytes expressing AAD-IIC-154 and AAD-IIC-222, labelled with the fluorophore MTS-TAMRA

A) Representative VCF recordings from the same oocyte expressing AAD-IIC-154 for the voltage step protocol shown and the superfusion conditions indicated. All data traces were baseline-adjusted to the steady-state fluorescence level at $V_h = -60$ mV.

B) AAD-IIC-154: *Upper*: $\Delta F-V$ data for different external $[\text{Na}^+]$. Data were offset to ΔF at the depolarizing limit predicted from a fit with Eqn 2 and normalised to F_{max} determined for 100 mM Na^+ . Continuous lines are refits to the adjusted data using Eqn 2. Data pooled from 4 oocytes. *Centre*: $\Delta F-V$ data for different $[\text{P}_i]$ in the presence of 100 mM Na^+ . Data were offset to ΔF at the depolarizing limit predicted from a fit with Eqn 2 and normalised to F_{max} determined for 0 mM P_i . Continuous lines are refits to the adjusted data using Eqn 2. Data pooled from 4 oocytes. *Lower*: Midpoint voltage ($V_{0.5}$) plotted as a function of $[\text{P}_i]$ derived from Boltzmann fit. Continuous line is fit using a modified form of Eqn 1 ($n_H=1$)

C) AAD-IIC-222: *Upper*: $\Delta F-V$ data for different external $[\text{Na}^+]$. Data were offset to ΔF at the depolarizing limit predicted from a fit with Eqn 2 and normalised to F_{max} determined for 100 mM Na^+ . Continuous lines are refits to the adjusted data using Eqn 2. Data pooled from 4 oocytes. *Centre*: $\Delta F-V$ data for different $[\text{P}_i]$ in the presence of 100 mM Na^+ . Data were offset to ΔF at the depolarizing limit predicted from a fit with Eqn 2 and normalised to F_{max} determined for 0 mM P_i . Continuous lines are refits to the adjusted data using Eqn 2. Data pooled from 4 oocytes. *Lower*: Midpoint voltage ($V_{0.5}$) plotted as a function of $[\text{P}_i]$ derived from Boltzmann fit. Continuous line is fit using a modified form of Eqn 1 ($n_H=1$).

Figure 9. Simulations using a reduced 7-state model recapitulate AAD-IIC kinetic behaviour.

A) Reduced 7-state model used for AAD-IIC simulations. Boxed values indicate effective valence associated with partial reactions representing empty carrier and Na^+ interactions and should be compared with the equivalent for the WT electrogenic transport cycle (Fig 1A). Transition (2 \leftrightarrow 9) accounts for uncoupled Na^+ -leak (see (1)). The voltage dependence of these partial reactions was modelled by applying Eyring Transition State Theory to describe the dependence of transition rate constants on membrane potential. The voltage-dependent partial reactions involve the movement of the equivalent lumped charges that result in a conformational state change by crossing a sharp energy barrier. We assumed symmetrical barriers, except for transition 9 \leftrightarrow 0 where an asymmetry factor ($asf = 0.15$) was used to improve agreement with experimental data. The rate constants (in s^{-1}) were as follows: $k_{10}=40\exp(-V_{z_{10}}\mu/2)$, $k_{01}=40\exp(V_{z_{10}}\mu/2)$; $k_{12}=[\text{Na}]_o 5000\exp(-V_{z_{12}}\mu/2)$, $k_{21}=1200\exp(V_{z_{12}}\mu/2)$, $k_{23}=[\text{Na}]_o 5000$, $k_{32}=400$, $k_{35}=[\text{Na}]_o [\text{P}_i]_o 2000$, $k_{53}=100$, $k_{56}=k_{65}=10$, $k_{69}=10^4$, $k_{29}=1$, $k_{09}=[\text{Na}]_i 200\exp(V_{z_{09}}\mu*asf)$, $k_{90}=7\exp(-V_{z_{09}}\mu(1-asf))$ and $\mu=e/kT \approx 40 \text{ V}^{-1}$ at 20 °C, $z_{10}=0.4$, $z_{12}=0.1$, $z_{09}=0.5$. The rates constants k_{96} , k_{92} were defined independently in terms of the other rate constants under conditions of zero driving force to satisfy the detailed balance for the leak and cotransport cycles. We assumed the following substrate concentrations on the cytosolic side: $[\text{Na}]_i = 10$ mM and $[\text{P}_i]_i = 1$ μM .

B) Simulations of presteady-state currents in response to voltage steps from -60 mV to test potentials as indicated for 3 superfusion conditions. In each case, baseline correction has been made. To obtain the true relaxation current magnitude (in B), traces should be scaled by $N_t e$, where N_t is the number of transporters and $e =$ the electronic charge. For each case, the corresponding traces from the representative oocyte (Fig 4A) are shown (*boxed*) for test potentials to -180 mV and +80 mV with the endogenous oocyte charging transients

removed by curve fitting. The measured data were scaled vertically to give a reasonable match to the simulated data.

C) *Upper*: Normalised Q - V data for Na^+ -interaction with external $\text{P}_i = 0$. Continuous lines are fits to data using Eqn 2 with all parameters unconstrained. Q - V data were normalised to Q_{max} obtained from fit to the data set for 100 mM Na^+ . *Lower*: Dependence $V_{0.5}$ on $[\text{Na}^+]$. This should be compared with Fig 4F.

D) *Upper*: Normalised Q - V data for P_i -interaction with external $[\text{Na}^+] = 100$ mM. Continuous lines are fits to data using Eqn 2 with all parameters unconstrained. Q - V data were normalised to Q_{max} obtained from fit to 0 mM P_i data set. *Lower*: Dependence of $V_{0.5}$ on $[\text{P}_i]$. This should be compared with Fig 5C. Continuous line is a fit using a modified form of Eqn 1 ($n_H=1$), giving $K_{0.5}^{\text{P}_i} = 1$ mM.

E) Simulated steady-state P_i -activation (-60 mV, 100 mM Na^+) (*filled symbols*) and Na^+ -activation (-60 mV, 1 mM P_i) (*open symbols*) using data set in B). The apparent affinity constants under these conditions were $K_{0.5}^{\text{P}_i} = 1.05$ mM and $K_{0.5}^{\text{Na}} = 102$ mM and the Hill coeff, (n_H) = 2.4, for Na^+ -activation found by fitting simulated data points with Eqn 1. Data should be scaled by $N_i e$ to obtain the P_i -induced current.

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References

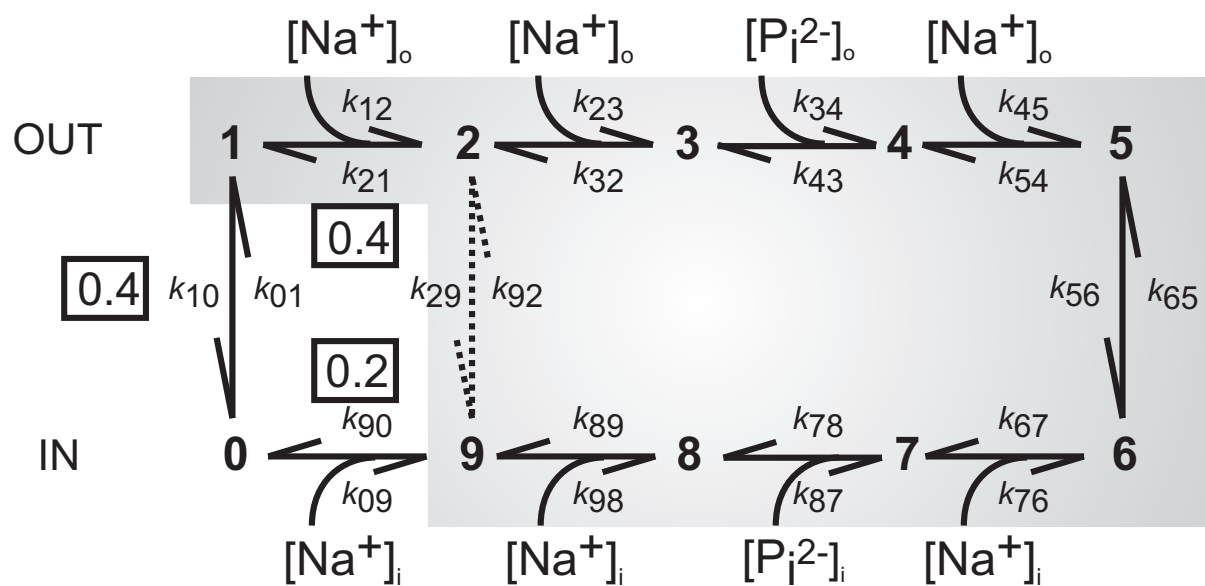
1. Andrini O, Ghezzi C, Murer H, Forster IC (2008) The leak mode of type II Na(+)-P(i) cotransporters. *Channels (Austin)* 2:346-357
2. Andrini O, Meinild AK, Ghezzi C, Murer H, Forster IC (2012) Lithium interactions with Na+-coupled inorganic phosphate cotransporters: insights into the mechanism of sequential cation binding. *Am J Physiol Cell Physiol* 302:C539-554
3. Bacconi A, Ravera S, Virkki LV, Murer H, Forster IC (2007) Temperature-dependency of steady-state and presteady-state kinetics of a type IIb Na⁺/P_i cotransporter. *Journal of Membrane Biology* in press
4. Bacconi A, Virkki LV, Biber J, Murer H, Forster IC (2005) Renouncing electrogenicity is not free of charge: switching on electrogenicity in a Na⁺-coupled phosphate cotransporter. *Proceedings of the National Academy of Sciences of the United States of America* 102:12606-12611
5. Binda F, Bossi E, Giovannardi S, Forlani G, Peres A (2002) Temperature effects on the presteady-state and transport-associated currents of GABA cotransporter rGAT1. *FEBS Lett* 512:303-307
6. Bossi E, Cherubino F, Margheritis E, Oyadeyi AS, Vollero A, Peres A (2012) Temperature effects on the kinetic properties of the rabbit intestinal oligopeptide cotransporter PepT1. *Pflugers Arch* 464:183-191
7. Breusegem SY, Takahashi H, Giral-Arnal H, Wang X, Jiang T, Verlander JW, Wilson P, Miyazaki-Anzai S, Sutherland E, Caldas Y, Blaine JT, Segawa H, Miyamoto K, Barry NP, Levi M (2009) Differential regulation of the renal sodium-phosphate cotransporters NaPi-IIa, NaPi-IIc, and PiT-2 in dietary potassium deficiency. *Am J Physiol Renal Physiol* 297:F350-361
8. Cha A, Bezanilla F (1998) Structural implications of fluorescence quenching in the Shaker K⁺ channel. *J Gen Physiol* 112:391-408
9. de la Horra C, Hernando N, Lambert G, Forster I, Biber J, Murer H (2000) Molecular determinants of pH sensitivity of the type IIa Na/P(i) cotransporter. *J Biol Chem* 275:6284-6287
10. Ehnes C, Forster IC, Bacconi A, Kohler K, Biber J, Murer H (2004) Structure-function relations of the first and fourth extracellular linkers of the type IIa Na⁺/P_i cotransporter: II. Substrate interaction and voltage dependency of two functionally important sites. *Journal of General Physiology* 124:489-503
11. Ehnes C, Forster IC, Kohler K, Bacconi A, Stange G, Biber J, Murer H (2004) Structure-function relations of the first and fourth predicted extracellular linkers of the type IIa Na⁺/P_i cotransporter: I. Cysteine scanning mutagenesis. *Journal of General Physiology* 124:475-488
12. Forrest LR, Kramer R, Ziegler C (2011) The structural basis of secondary active transport mechanisms. *Biochim Biophys Acta* 1807:167-188
13. Forster I, Hernando N, Biber J, Murer H (1998) The voltage dependence of a cloned mammalian renal type II Na⁺/P_i cotransporter (NaPi-2). *Journal of General Physiology* 112:1-18
14. Forster IC, Biber J, Murer H (2000) Proton-sensitive transitions of renal type II Na⁺-coupled phosphate cotransporter kinetics. *Biophysical Journal* 79:215-230
15. Forster IC, Hernando N, Biber J, Murer H (2012) Phosphate Transport Kinetics and Structure-Function Relationships of SLC34 and SLC20 Proteins. *Curr Top Membr* 70:313-356
16. Forster IC, Kohler K, Biber J, Murer H (2002) Forging the link between structure and function of electrogenic cotransporters: the renal type IIa Na⁺/P_i cotransporter as a case study. *Progress in Biophysics and Molecular Biology* 80:69-108
17. Forster IC, Loo DD, Eskandari S (1999) Stoichiometry and Na⁺ binding cooperativity of rat and flounder renal type II Na⁺-P_i cotransporters. *American Journal of Physiology* 276:F644-649
18. Forster IC, Wagner CA, Busch AE, Lang F, Biber J, Hernando N, Murer H, Werner A (1997) Electrophysiological characterization of the flounder type II Na⁺/P_i cotransporter (NaPi-5) expressed in *Xenopus laevis* oocytes. *Journal of Membrane Biology* 160:9-25
19. Ghezzi C, Meinild AK, Murer H, Forster IC (2011) Voltage- and substrate-dependent interactions between sites in putative re-entrant domains of a Na(+)-coupled phosphate cotransporter. *Pflugers Arch* 461:645-663
20. Ghezzi C, Murer H, Forster IC (2009) Substrate interactions of the electroneutral Na+-coupled inorganic phosphate cotransporter (NaPi-IIc). *J Physiol* 587:4293-4307
21. Hazama A, Loo DD, Wright EM (1997) Presteady-state currents of the rabbit Na⁺/glucose cotransporter (SGLT1). *J Membr Biol* 155:175-186
22. Karlin A, Akabas MH (1998) Substituted-cysteine accessibility method. *Methods in Enzymology* 293:123-145
23. Lambert G, Forster IC, Biber J, Murer H (2000) Cysteine residues and the structure of the rat renal proximal tubular type II sodium phosphate cotransporter (rat NaPi IIa). *Journal of Membrane Biology* 176:133-141
24. Lambert G, Forster IC, Stange G, Biber J, Murer H (1999) Properties of the mutant Ser-460-Cys implicate this site in a functionally important region of the type IIa Na⁺/P_i cotransporter protein. *Journal of General Physiology* 114:637-652

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25. Lambert G, Forster IC, Stange G, Kohler K, Biber J, Murer H (2001) Cysteine mutagenesis reveals novel structure-function features within the predicted third extracellular loop of the type IIa Na⁺/P_i cotransporter. *Journal of General Physiology* 117:533-546
 26. Lester HA, Mager S, Quick MW, Corey JL (1994) Permeation properties of neurotransmitter transporters. *Annu Rev Pharmacol Toxicol* 34:219-249
 27. Mackenzie B, Loo DD, Wright EM (1998) Relationships between Na⁺/glucose cotransporter (SGLT1) currents and fluxes. *J Membr Biol* 162:101-106
 28. Matthews E, Jr., Rahnama-Vaghef A, Eskandari S (2009) Inhibitors of the gamma-aminobutyric acid transporter 1 (GAT1) do not reveal a channel mode of conduction. *Neurochem Int* 55:732-740
 29. Meinild AK, Forster IC (2012) Using lithium to probe sequential cation interactions with GAT1. *Am J Physiol Cell Physiol* 302:C1661-1675
 30. Radanovic T, Gislser SM, Biber J, Murer H (2006) Topology of the type IIa Na⁺/P_i cotransporter. *J Membr Biol* 212:41-49
 31. Segawa H, Kaneko I, Takahashi A, Kuwahata M, Ito M, Ohkido I, Tatsumi S, Miyamoto K (2002) Growth-related renal type II Na/Pi cotransporter. *Journal of Biological Chemistry* 277:19665-19672
 32. Virkki LV, Forster IC, Bacconi A, Biber J, Murer H (2005) Functionally important residues in the predicted 3rd transmembrane domain of the type IIa sodium-phosphate co-transporter (NaPi-IIa). *Journal of Membrane Biology* 206:227-238
 33. Virkki LV, Forster IC, Biber J, Murer H (2005) Substrate interactions in the human type IIa sodium-phosphate cotransporter (NaPi-IIa). *American Journal of Physiology* 288:F969-F981
 34. Virkki LV, Murer H, Forster IC (2006) Mapping conformational changes of the type IIb Na⁺/P_i cotransporter by voltage clamp fluorometry. *Journal of Biological Chemistry* 281:28837-28849
 35. Virkki LV, Murer H, Forster IC (2006) Voltage clamp fluorometric measurements on a type II Na⁺-coupled P_i cotransporter: shedding light on substrate binding order. *Journal of General Physiology* 127:539-555
 36. Werner A, Kinne RK (2001) Evolution of the Na-P_i cotransport systems. *American Journal of Physiology* 280:R301-312
 37. Winzor DJ, Jackson CM (2006) Interpretation of the temperature dependence of equilibrium and rate constants. *J Mol Recognit* 19:389-407

Figure 1

Figure 1

A



B

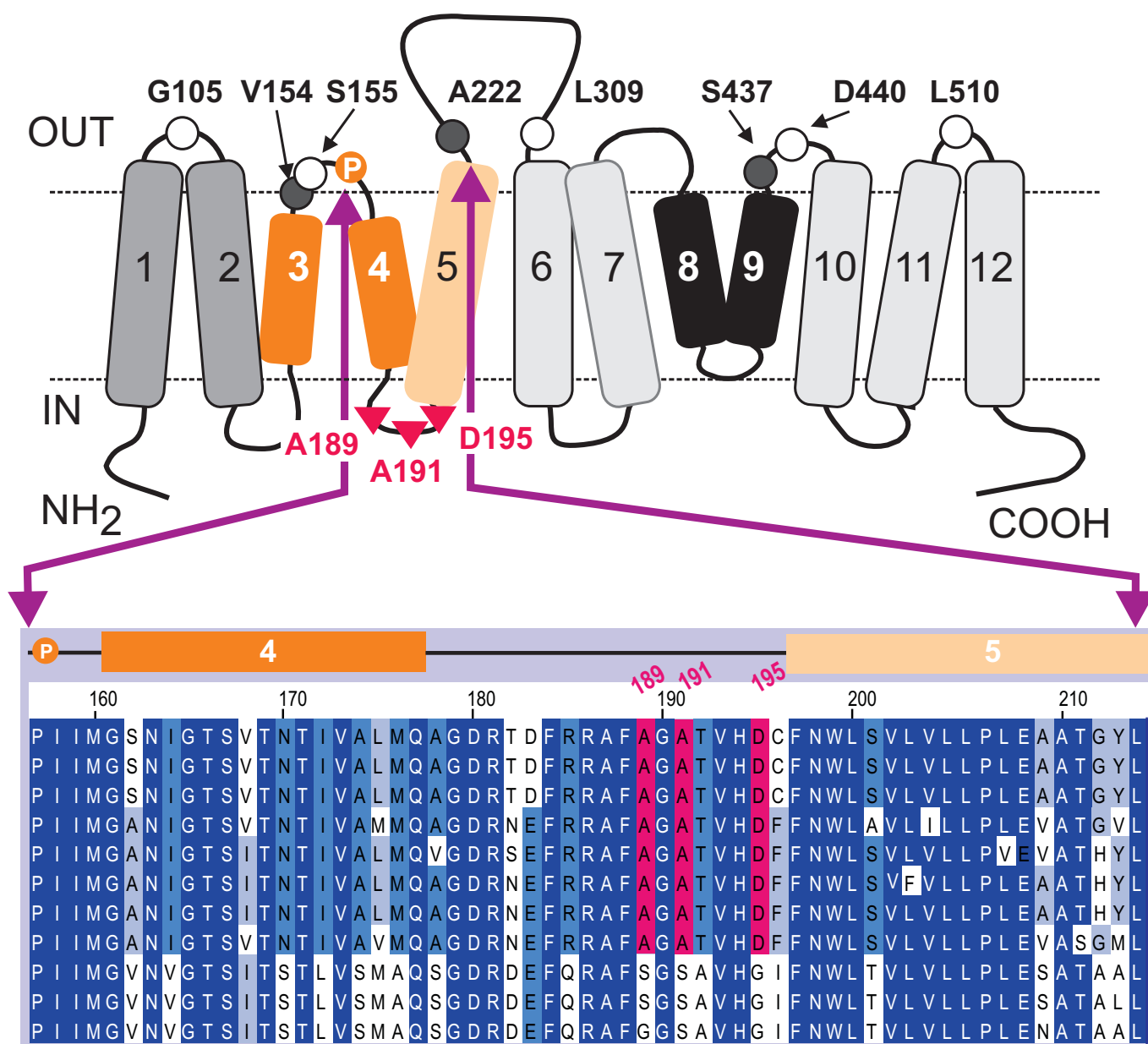
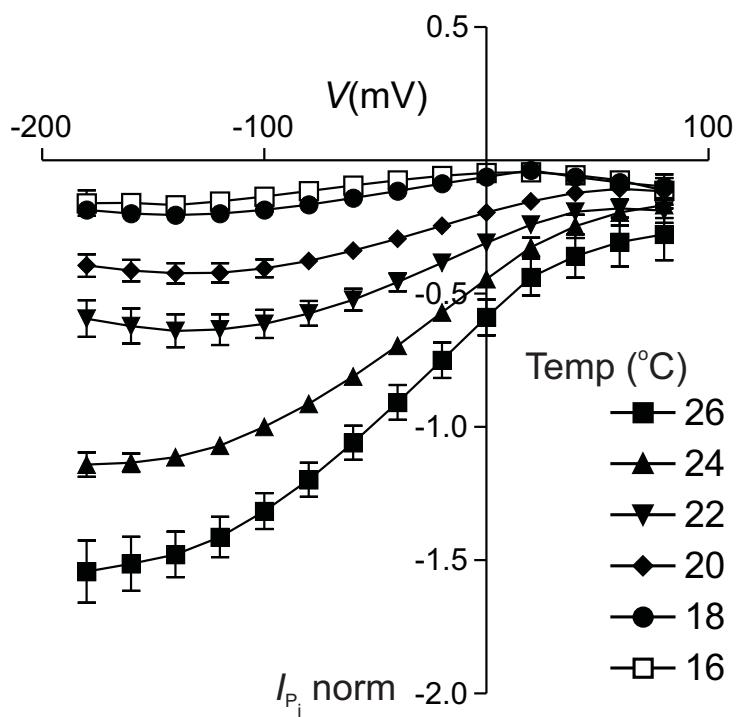
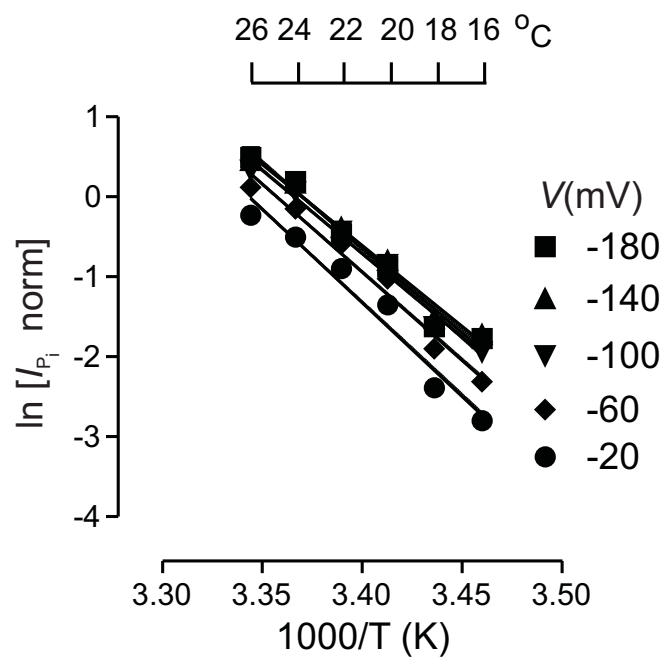


Figure 2

A



B



C

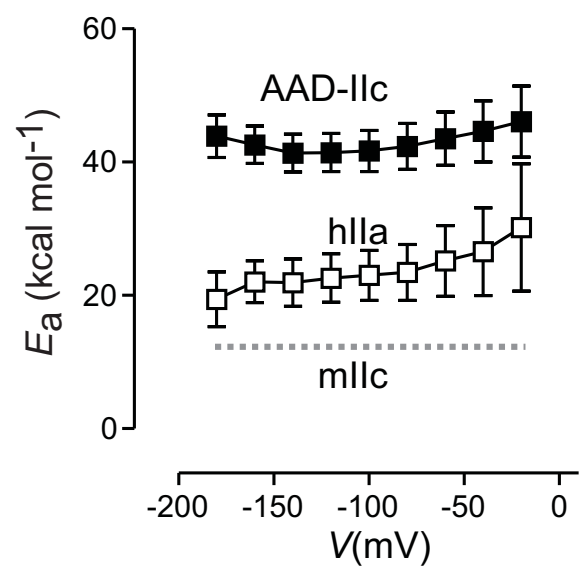
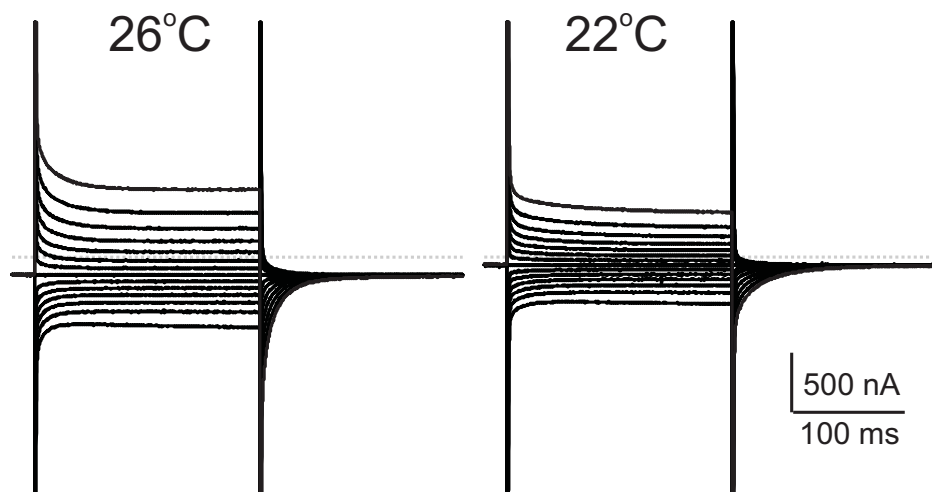


Figure 2

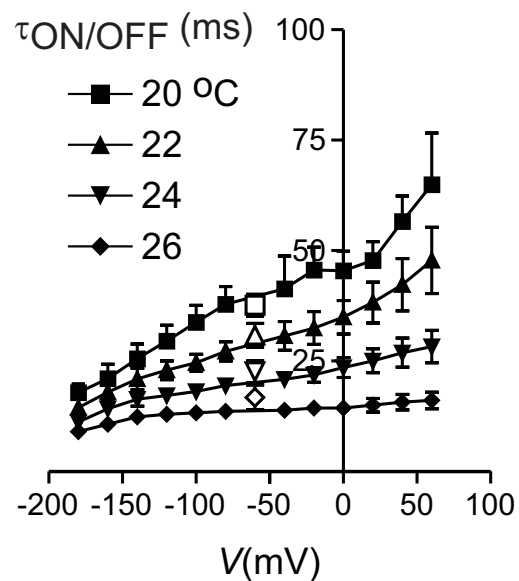
Figure 3

Figure 3

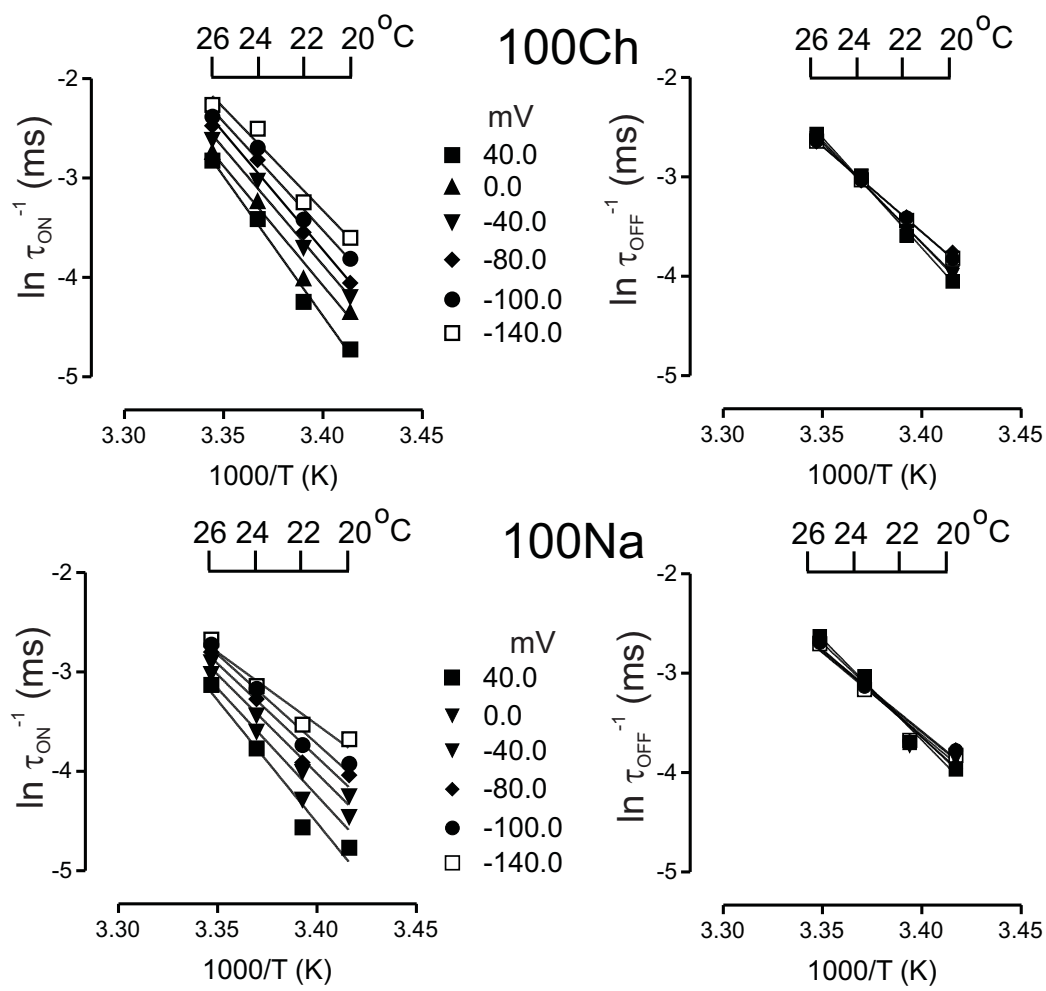
A



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D

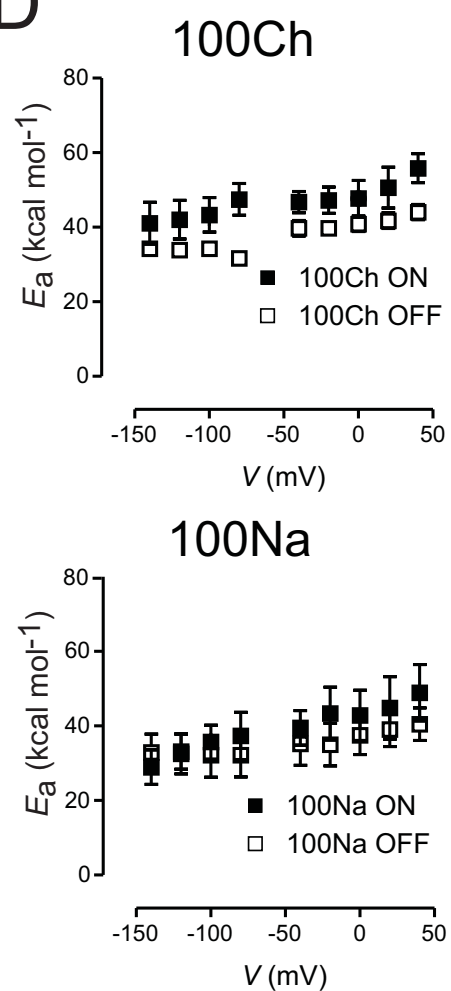


Figure 4

Figure 4

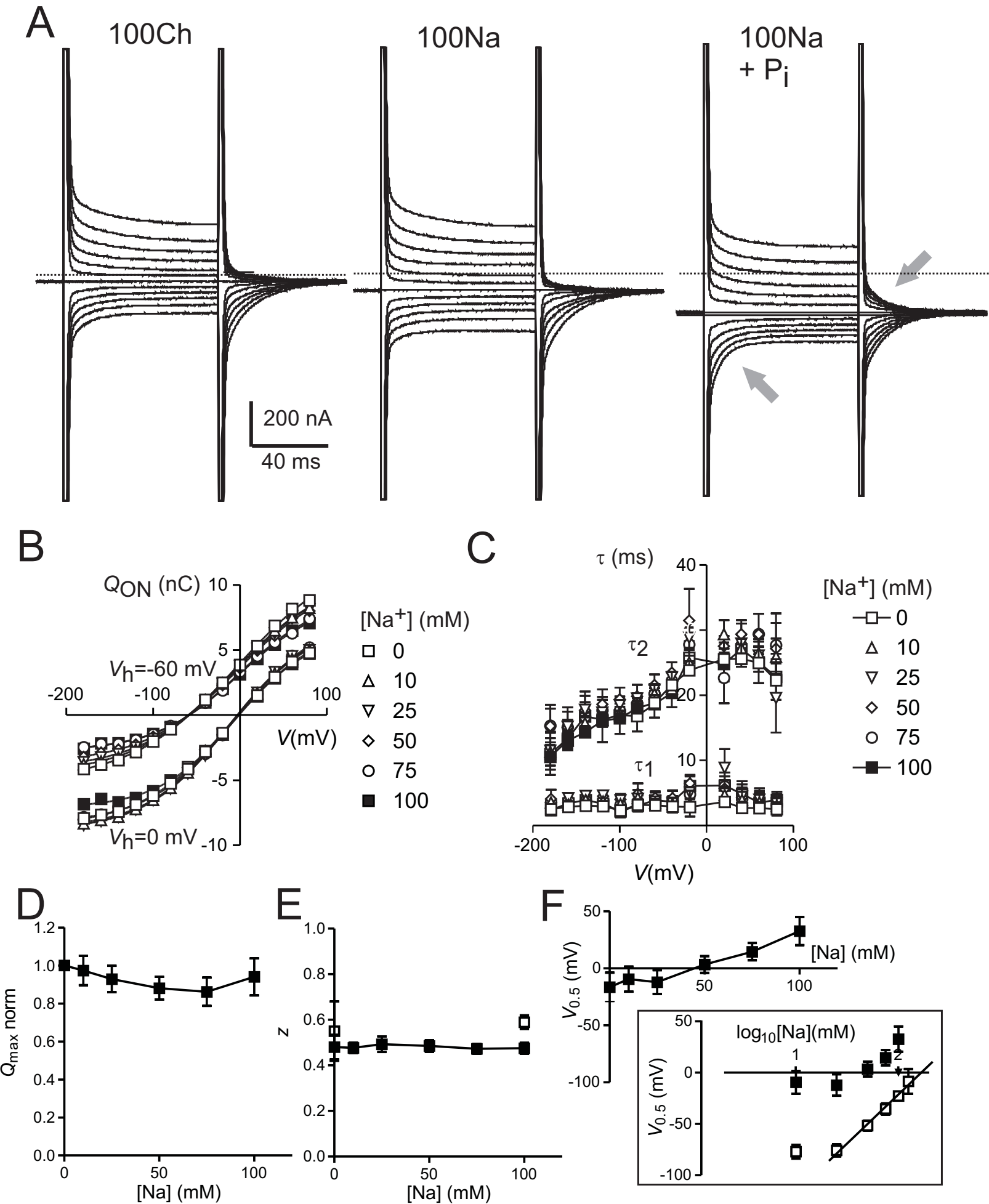
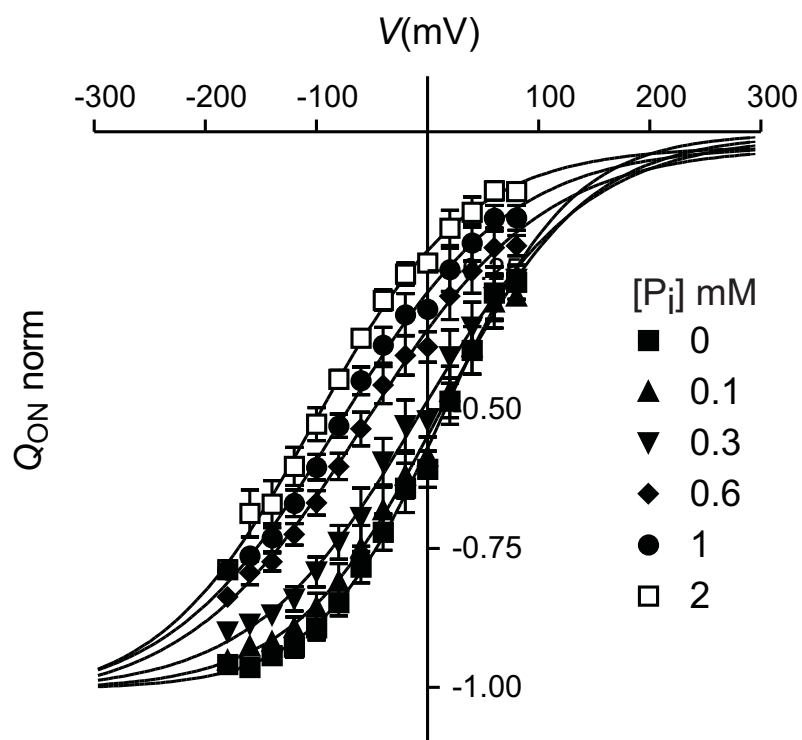
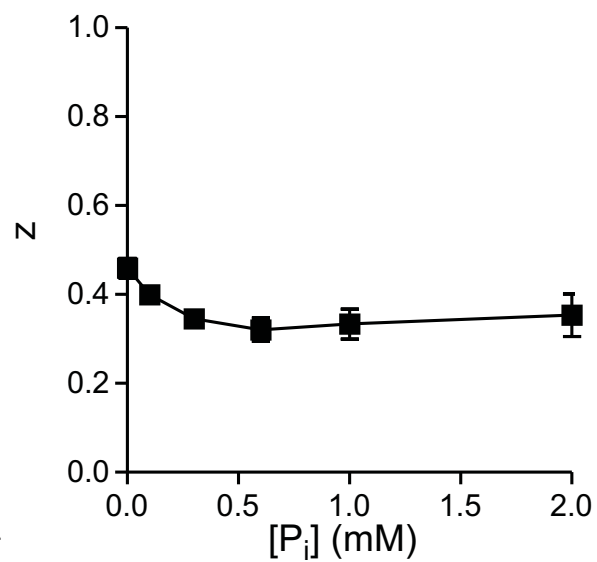


Figure 5

A



B



C

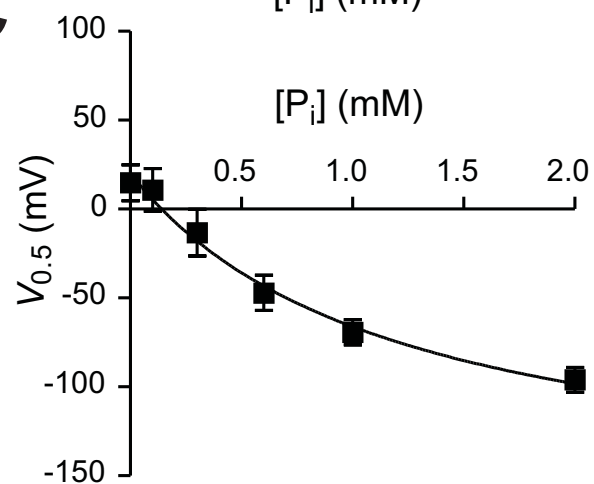
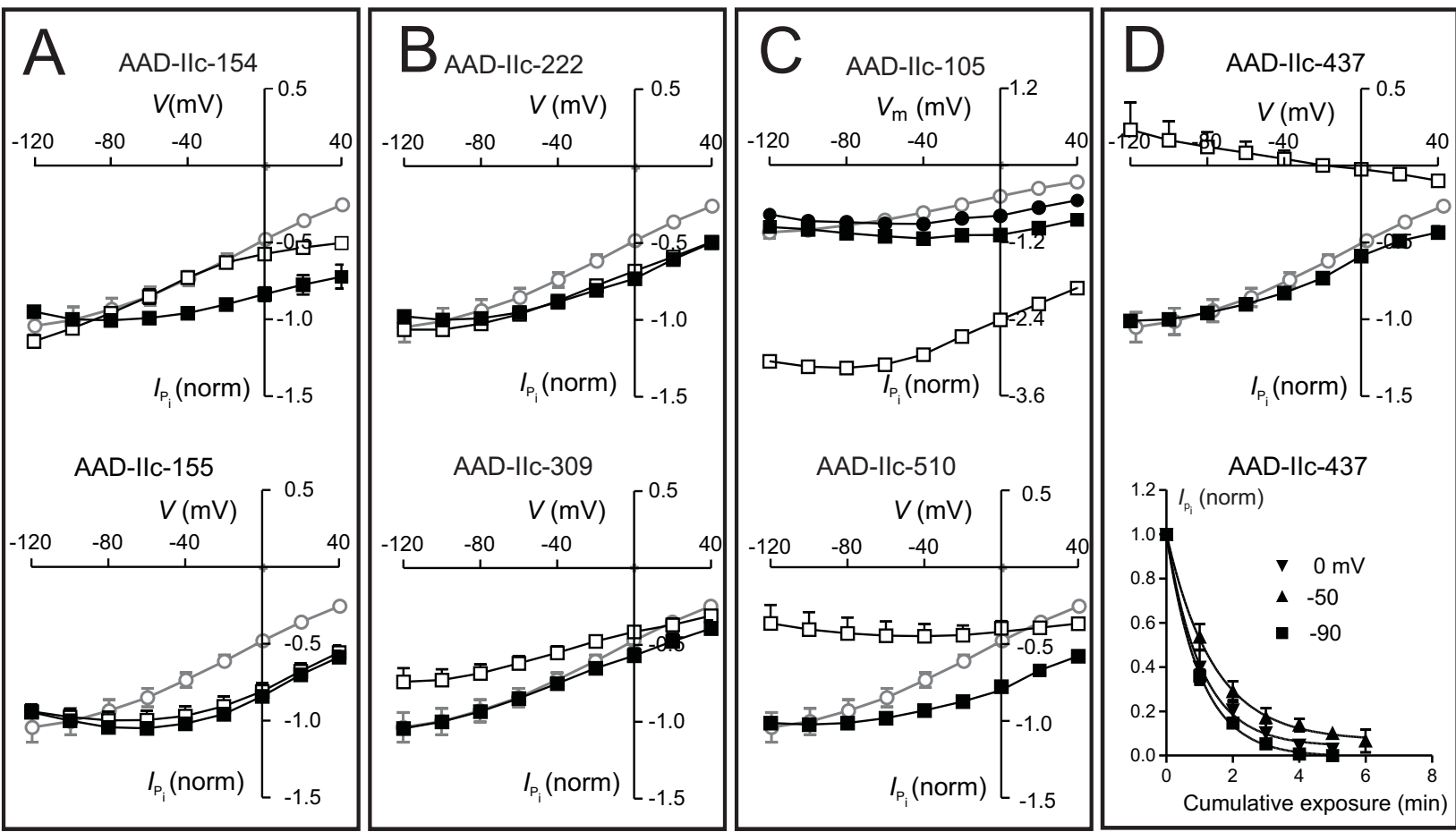


Figure 5

Figure 6

Figure 6



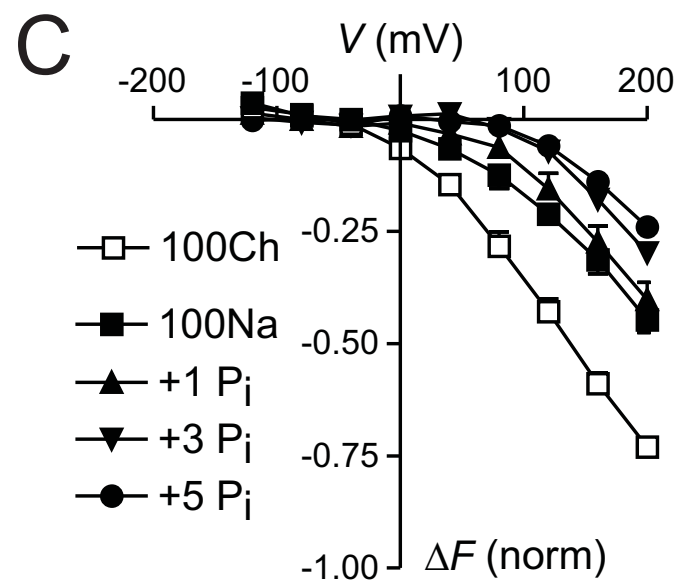
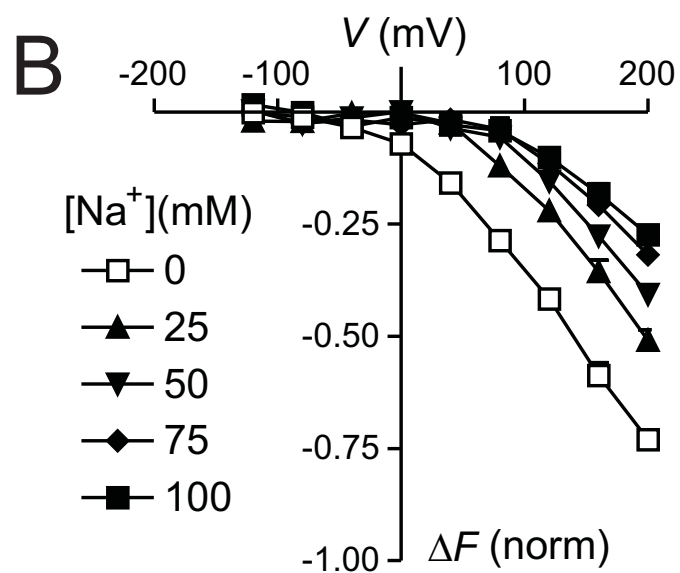
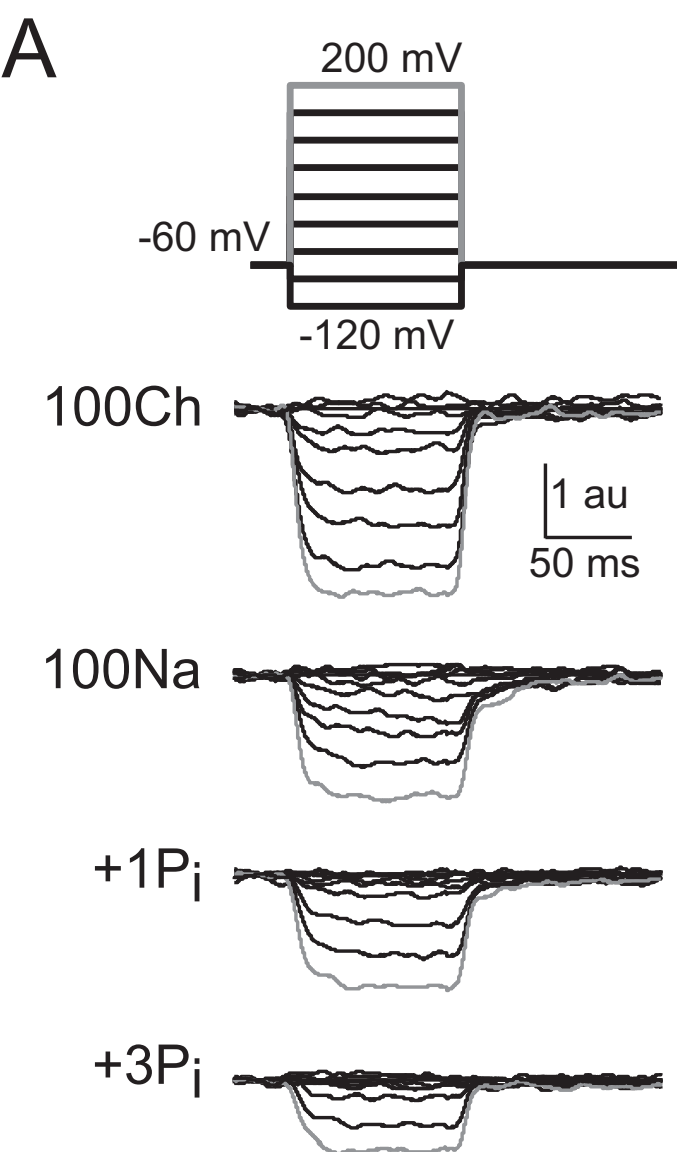


Figure 8

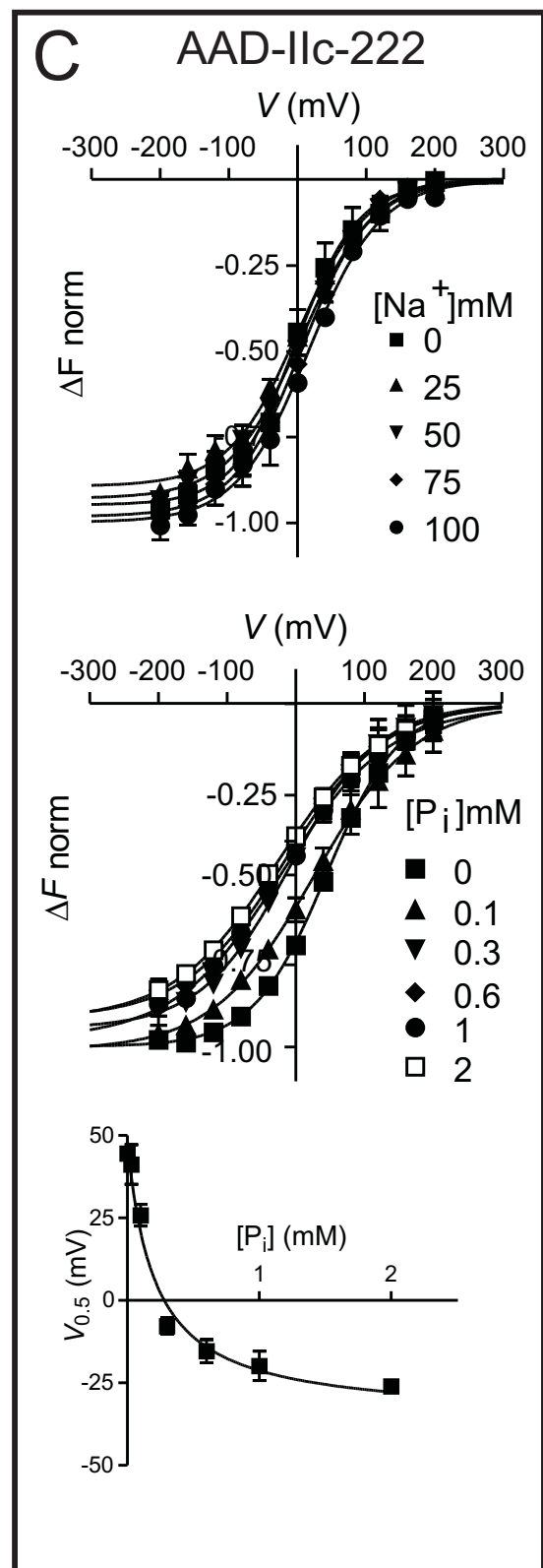
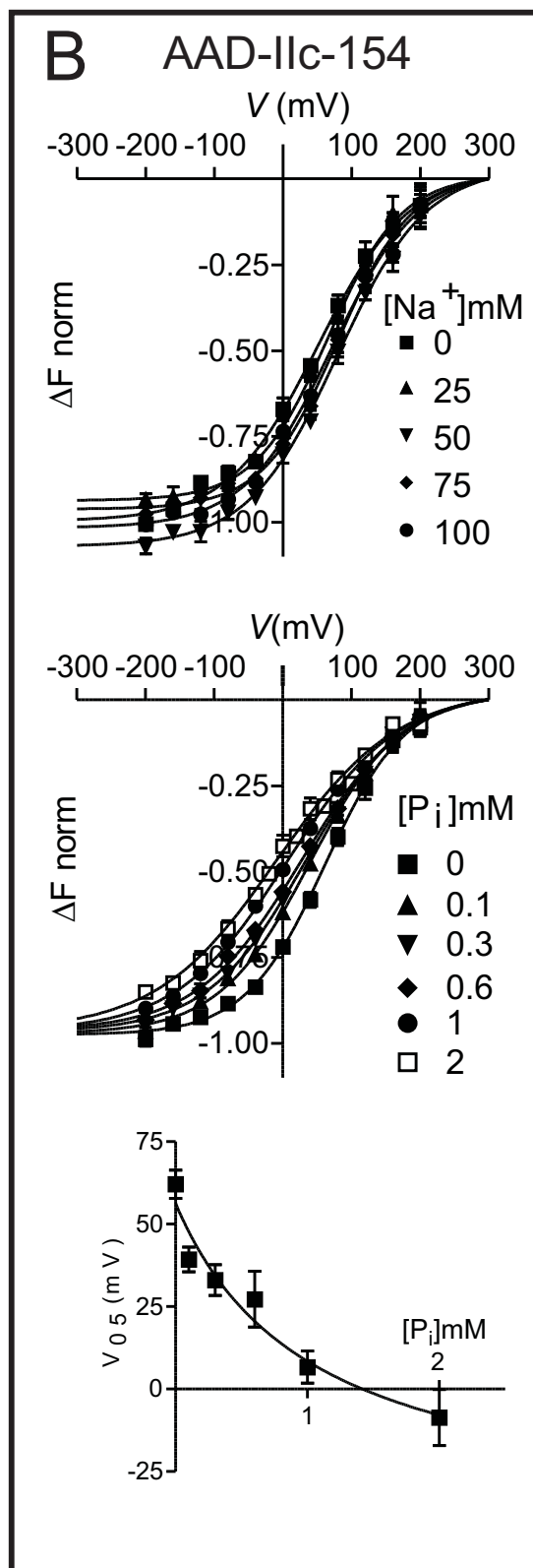
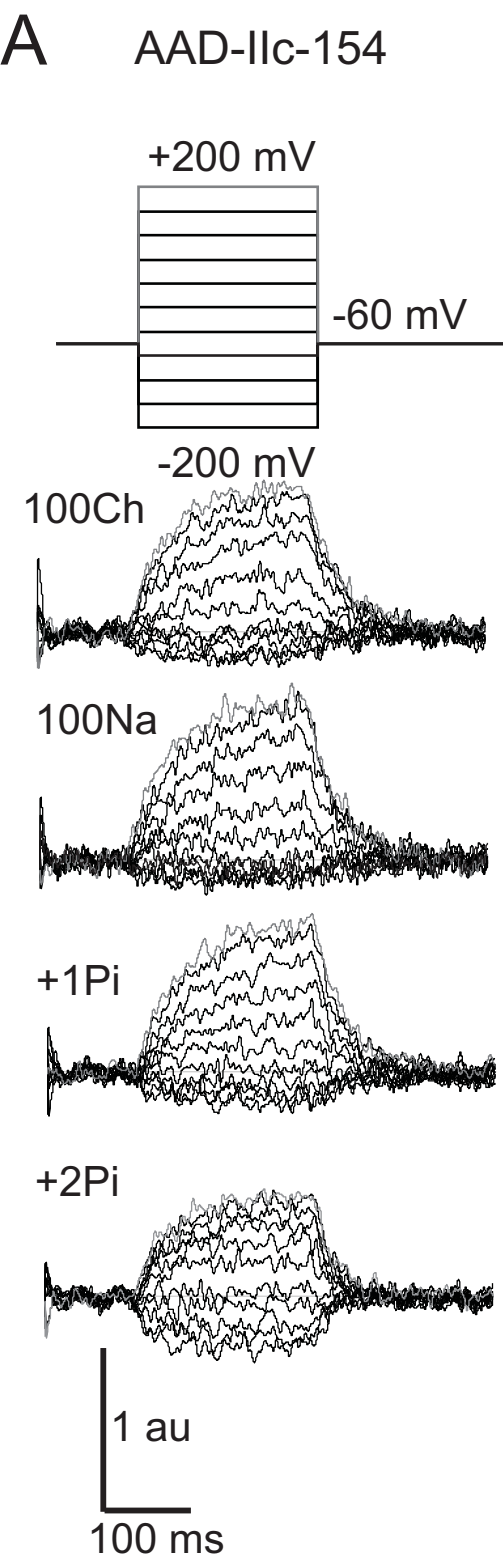


Figure 8

Figure 9

Figure 9

