Residues Important for Nitrate/Proton Coupling in Plant and Mammalian CLC Transporters*

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Members of the CLC gene family either function as chloride channels or as anion/proton exchangers. The plant AtClC-a uses the pH gradient across the vacuolar membrane to accumulate the nutrient NO$_3^-$ in this organelle. When AtClC-a was expressed in Xenopus oocytes, it mediated NO$_3^-$/$H^+$ exchange and less efficiently mediated CI$^-$/H$^+$ exchange. Mutating the “gating glutamate” Glu-203 to alanine resulted in an uncoupled anion conductance that was larger for CI$^-$ than NO$_3^-$ . Replacing the “proton glutamate” Glu-270 by alanine abolished currents. These could be restored by the uncoupling E203A mutation. Whereas mammalian endosomal CIC-4 and CIC-5 mediate stoichiometrically coupled 2CI$^-$/H$^+$ exchange, their NO$_3^-$ transport is largely uncoupled from protons. By contrast, the AtClC-a-mediated NO$_3^-$ accumulation in plant vacuoles requires tight NO$_3^-$/$H^+$ coupling. Comparison of AtClC-a and CIC-5 sequences identified a proline in AtClC-a that is replaced by serine in all mammalian CLC isoforms. When this proline was mutated to serine (P160S), CI$^-$/H$^+$ exchange of AtClC-a proceeded as efficiently as NO$_3^-$/$H^+$ exchange, suggesting a role of this residue in NO$_3^-$/$H^+$ exchange. Indeed, when the corresponding serine of CIC-5 was replaced by proline, this CI$^-$/H$^+$ exchanger gained efficient NO$_3^-$/$H^+$ coupling. When inserted into the model Torpedo chloride channel CIC-0, the equivalent mutation increased nitrate relative to chloride conductance. Hence, proline in the CIC pore signature sequence is important for NO$_3^-$/$H^+$ exchange and NO$_3^-$ conductance both in plants and mammals. Gating and proton glutamates play similar roles in bacterial, plant, and mammalian CLC anion/proton exchangers.

CLC proteins are found in all phyla from bacteria to humans and either mediate electrogenic anion/proton exchange or function as chloride channels (1). In mammals, the roles of plasma membrane CLC CI$^-$ channels include transepithelial transport (2–5) and control of muscle excitability (6), whereas vesicular CLC exchangers may facilitate endocytosis (7) and lysosomal function (8–10) by electrically shunting vesicular proton pump currents (11). In the plant Arabidopsis thaliana, there are seven CLC isoforms (AtCIC-a–AtCIC-g) (12–15), which may mostly reside in intracellular membranes. AtCIC-a uses the pH gradient across the vacuolar membrane to transport the nutrient nitrate into that organelle (16). This secondary active transport requires a tightly coupled NO$_3^-$/$H^+$ exchange. Astonishingly, however, mammalian CIC-4 and -5 and bacterial EcCIC-1 (one of the two CLC isoforms in Escherichia coli) display tightly coupled CI$^-$/H$^+$ exchange, but anion flux is largely uncoupled from H$^+$ when NO$_3^-$ is transported (17–21).

The lack of appropriate expression systems for plant CLC transporters (12) has so far impeded structure-function analysis that may shed light on the ability of AtCIC-a to perform efficient NO$_3^-$/$H^+$ exchange. This dearth of data contrasts with the extensive mutagenesis work performed with CLC proteins from animals and bacteria. The crystal structure of bacterial CLC homologues (22, 23) and the investigation of mutants (17, 19–21, 24–29) have yielded important insights into their structure and function. CLC proteins form dimers with two largely independent permeation pathways (22, 25, 30, 31). Each of the monomers displays two anion binding sites (22). A third binding site is observed when a certain key glutamate residue, which is located halfway in the permeation pathway of almost all CLC proteins, is mutated to alanine (23). Mutating this gating glutamate in CLC CI$^-$ channels strongly affects or even completely suppresses single pore gating (23), whereas CLC exchangers are transformed by such mutations into pure anion conductances that are not coupled to proton transport (17, 19, 20). Another key glutamate, located at the cytoplasmic surface of the CLC monomer, seems to be a hallmark of CLC anion/proton exchangers. Mutating this proton glutamate to nontitratable amino acids uncouples anion transport from protons in the bacterial EcCIC-1 protein (27) but seems to abolish transport altogether in mammalian CIC-4 and -5 (21). In those latter proteins, anion transport could be restored by additionally introducing an uncoupling mutation at the gating glutamate (21).

The functional complementation by AtCIC-c and -d (12, 32) of growth phenotypes of a yeast strain deleted for the single

The abbreviations used are: AtCIC-n, member n of the CLC family of CI$^-$ channels; AtCIC-c and -d transporters in the plant Arabidopsis thaliana; CIC-n, member n of the CLC family of chloride channel and transporters (in animals); pH$_i$, intracellular pH; pH$_e$, extracellular pH; [NO$_3^-$], and [CI$^-$], current in the presence of NO$_3^-$ and CI$^-$, respectively, which in CLC exchangers also involves an H$^+$ component; WT, wild-type; MES, 2-(N-morpholino)ethane-sulfonic acid; BECF, 2,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein.

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yeast CLC Gef1 (33) suggested that these plant CLC proteins function in anion transport but could not reveal details of their biophysical properties. We report here the first functional expression of a plant CLC in animal cells. Expression of wild-type (WT) and mutant AtClC-a in *Xenopus* oocytes indicate a general role of gating and proton glutamate residues in anion/proton coupling across different isoforms and species. We identified a proline in the CLC signature sequence of AtClC-a that plays a crucial role in NO$_3^-$ /H$^+$ exchange. Mutating it to serine, the residue present in mammalian CLC proteins at this position, rendered AtClC-a Cl$^-$ /H$^+$ exchange as efficient as NO$_3^-$ /H$^+$ exchange. Conversely, changing the corresponding serine of ClC-5 to proline converted it into an efficient NO$_3^-$ /H$^+$ exchanger. When proline replaced the critical serine in *Torpedo* CiC-0, the relative NO$_3^-$ conductance of this model Cl$^-$ channel was drastically increased, and “fast” protopore gating was slowed.

**EXPERIMENTAL PROCEDURES**

*Molecular Biology—*cDNAs of *A. thaliana* AtClC-a (12), rat ClC-5 (34), and *Torpedo marmorata* CiC-0 (35) were cloned into the pTLN (36) expression vector. Mutations were generated by recombinant PCR and confirmed by sequencing. Capped cRNA was transcribed from linearized plasmids using the Ambion mMESSAGE mMACHINE kit (SP6 RNA polymerase) for pTLN according to the manufacturer’s instructions.

*Expression in Xenopus Oocyte and Two-electrode Voltage-Clamp Studies—*Pieces of ovary were obtained by surgery from deepanesthetized (0.1% tricaine; Sigma) pigmented or albino *Xenopus laevis* frogs. Oocytes were prepared by manual dissection and collagenase A (Roche Applied Science) digestion. 23 ng (ClC-5), 46–50 ng (AtClC-a), or 1–3 ng (ClC-0) of cRNA were injected into oocytes. Oocytes were kept in ND96 solution (containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, pH 7.5) at 17 °C for 1–2 days (ClC-0), 3–4 days (ClC-5), or for 5–6 days (AtClC-a). Two-electrode voltage clamping was performed at room temperature (20–24 °C) using a TEC10 amplifier (npi Electronics, Tamm, Germany) and pClamp9 software (Molecular Devices). The standard bath solution contained 96 mM NaCl, 2 mM K$^+$ gluconate, 5 mM Ca$^{2+}$-D-gluconate, 1.2 mM MgSO$_4$, 5 mM HEPES, pH 7.5 (or MES for buffering to pH 5.5 or 6.5; Tris for pH 8.5). For some experiments, NaCl was substituted with equal amounts of either NaNO$_3$, NaBr, or NaI. Ag/AgCl electrodes and 3 mM KCl agar bridges were used as reference and bath electrodes, respectively.

*Measurements of Relative Intracellular pH Changes Using the “Fluorocyte” Device—*Proton transport was measured semi-quantitatively by monitoring intracellular fluorescence signal changes using the Fluorocyte device (21). Briefly, 23 nl of saturated aqueous solution of the pH indicator BCECF (Molecular Probes) were injected into oocytes 10–30 min before measurements. Oocytes were placed over a hole 0.8 mm in diameter, through which BCECF fluorescence changes were measured in response to pulse trains, which served to reduce the possible activation of endogenous oocyte currents. Starting from a holding voltage of −60 mV, depolarizing pulse trains clamped oocytes to +90 mV for 400 ms and −60 mV for 100 ms, whereas hyperpolarizing pulse trains started from −30 mV and clamped to −160 mV for 400 ms and to −30 mV for 100 ms. BCECF fluorescence was measured with a photodiode and digitally Bessel-filtered at 0.3 Hz. These nonratiometric measurements generally show drifts owed to bleaching or intracellular dye distribution (21) but allow for sensitive measurements of pH changes upon changes in voltage or external ion composition.

**RESULTS**

Previous attempts to functionally express plant CLC proteins in animal cells proved unsuccessful (12). However, when *Xenopus* oocytes were measured 5 or more days after injecting AtClC-a cRNA, currents well above background levels were observed in two-electrode voltage-clamp experiments (Fig. 1). These outwardly rectifying currents were roughly 30% larger when extracellular chloride was replaced by nitrate (NO$_3^-$) (Fig. 1, A–C), smaller with extracellular iodide, and nearly unchanged with a replacement by bromide (Fig. 1C). Reversal potentials indicated that the apparent anion permeability was larger for NO$_3^-$ than for the other anions tested. It should be noted that for CLC exchangers, the observed apparent permeabilities and conductances represent those of coupled anion/proton exchange rather than diffusive anion transport. Contrasting with the slow activation of currents by depolarization observed in plant vacuoles (16), heterologously expressed AtClC-a currents almost totally lacked time-dependent relaxations (Fig. 1, A and B). Like currents elicited by ClC-4 or -5 (26), AtClC-a currents were reduced by acidic extracellular pH (pH$_a$) (Fig. 1, D–F). With extracellular NO$_3^-$, the extracellular pH had to be more acidic to obtain the same degree of current decrease as with Cl$^-$ (Fig. 1F).

We next explored proton transport of AtClC-a by measuring semiquantitatively the pH$_i$ of voltage-clamped oocytes using the Fluorocyte system (21). Net ion transport was elicited by strongly depolarizing oocytes to +90 mV. Because prolonged strong depolarization can elicit endogenous transport processes in oocytes, trains of depolarizing pulses were used instead (20, 21). An inside-positive voltage should lead to anion influx and proton efflux through electrogenic anion/proton exchangers. Pulsing AtClC-a-expressing oocytes to positive voltages indeed induced intracellular alkalinization (Fig. 1G). Importantly, alkalinization was also observed when protons were extruded against their electrochemical potential (at pH$_a$ 5.5), suggesting that their transport is driven by the coupled NO$_3^-$ /H$^+$ exchange (Fig. 1H). Under either condition (pH$_a$ 7.5 or 5.5), alkalinization occurred more rapidly with extracellular NO$_3^-$ than with Cl$^-$ (Fig. 1G). This shows that AtClC-a more efficiently mediates NO$_3^-$ /H$^+$ exchange than Cl$^-$ /H$^+$ exchange, a finding compatible with its role in accumulating NO$_3^-$ in plant vacuoles (16, 37). In contrast with ClC-4 and -5 (26), AtClC-a mediates robust currents also at negative voltages (Fig. 1, A–E). Accordingly, and in contrast to ClC-5 (see Fig. 5H), AtClC-a mediated proton influx when oocytes were pulsed to negative voltages (−160 mV) (Fig. 1J). Substituting extracellular Cl$^-$ by NO$_3^-$ failed to influence the rate of acidification because proton influx is coupled to an efflux of anions from the interior of oocytes.

In EcClC-1 and ClC-4 and -5, a certain gating glutamate is important for coupling chloride fluxes to proton countertrans-
port (17, 19, 20). In crystals of the bacterial protein, the negatively charged side chain of this glutamate seems to block the permeation pathway (22, 23). Mutating this glutamate to alanine in ClC-4 and -5 not only leads to flux uncoupling but also abolishes the strong outward rectification of either transporter (26). Likewise, currents of the E203A mutant lost their outward rectification (Fig. 2). Rather than being linear and again similar to the analogous mutants of ClC-4 and -5 (19, 20, 26), currents showed slight outward rectification at positive voltages and slight inward rectification at negative voltages. Contrasting with the moderate changes in ion selectivity observed with such mutations in CIC-4 and -5 (26), the E203A mutation drastically altered the anion selectivity of AtClC-a. Whereas reversal potentials indicated that the mutant remained more permeable for NO$_3^-$ than for Cl$^-$ (Fig. 2C, inset), its conductance was now reduced rather than increased when extracellular Cl$^-$ was replaced by NO$_3^-$ (Fig. 2, A–C; see Fig. 4F). With either external anion, currents were insensitive to changes in pH$_o$ (Fig. 2, D and E), and trains of depolarizing pulses failed to change pH$_i$ (Fig. 2F). Hence, the E203A mutant displayed uncoupled anion transport and drastically reduced conductance in the presence of NO$_3^-$.

We next studied the AtClC-a mutant E270A, which is equivalent to the proton glutamate mutations of EcClC-1 (27) and

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**FIGURE 1. Electrophysiological characterization of AtClC-a in Xenopus oocytes.** A and B, voltage-clamp traces of oocyte-expressed AtClC-a in either Cl$^-$-containing (A) or NO$_3^-$-containing (B) solutions. From a holding potential of $-30$ mV, the oocyte was clamped in 20-mV steps to voltages between $-100$ and $+100$ mV for 500 ms. C, steady-state I/V curves of AtClC-a with different extracellular anions. Currents were normalized for individual oocytes to a current at $+80$ mV in Cl$^-$ solution (with a mean value of $3.38 \pm 0.40$ nA). AtClC-a has a NO$_3^-$ > Br$^-$ > Cl$^-$ > I$^-$ conductance sequence (mean values from 13 oocytes from four batches; error bars, S.E.). Inset, higher magnification of the I/V curve to show reversal potentials. D and E, steady-state I/V curves of AtClC-a at different values of extracellular pH (pH$_o$) in Cl$^-$-containing (D) or NO$_3^-$-containing (E) solutions. The voltage-clamp protocol was performed as in A and B. Currents were normalized for individual oocytes to the respective current at $+80$ mV at pH 7.5 (five oocytes for each data point; error bars, S.E.). F, currents at $+80$ mV as a function of pH$_o$ with extracellular Cl$^-$ or NO$_3^-$ (shown in boxes above). Depolarizing pulses elicited intracellular alkalinization both with pH$_o$ 7.5 (G) and pH$_o$ 5.5, when proton transport occurs against a gradient (H). Extracellular NO$_3^-$ accelerates depolarization-driven alkalinization (G and H) but not hyperpolarization-driven acidification (I, pH$_o$ 7.5). Only changes in relative fluorescence (rel. fluor.) induced by changes in voltage or external anions are relevant because of the drift inherent to the Fluorocyte technique (21). Results as in G–I were obtained in 26, 6, and 7 independent experiments, respectively.
Nitrate/Proton Coupling in CLC Transporters

FIGURE 2. Impact of gating glutamate mutant E203A on AtClC-a properties. A and B, voltage-clamp traces of oocytes expressing the E203A mutant of AtClC-a with extracellular Cl$^\text{−}$/NO$_3^\text{−}$ (A) or NO$_3^\text{−}$/NO$_3^\text{−}$ (B) using the pulse protocol of Fig. 1 (A and B). C, steady-state I/V curves of AtClC-a(E203A) with different extracellular anions reveal a changed conductance sequence of Cl$^\text{−}$$>$Br$^\text{−}$$>$NO$_3^\text{−}$$>$I$^\text{−}$ (mean of 19 oocytes; error bars, S.E.). Currents were normalized to current in Cl$^\text{−}$ at +80 mV (with mean current of 3.05 ± 0.22 μA). Inset, higher magnification of the I/V curve to show reversal potentials. D and E, currents of AtClC-a(E203A) are insensitive to pH$_\text{ox}$, both in the presence of extracellular Cl$^\text{−}$ (D) or NO$_3^\text{−}$ (E). The number of oocytes is 10 for D and 14 for E (error bars, S.E.). F, lack of depolarization (depol.)-induced alkalinization with the E203A mutant. 25 independent experiments gave similar results. rel. fluoro, relative fluorescence.

CIC-4 and -5 (Fig. 3) (21). Neither currents (Fig. 3, A–C) nor depolarization-induced H$^+$ transport (Fig. 3, G and H) were different from background levels with this mutant. However, when combined with the uncoupling E203A mutation in the gating glutamate, the AtClC-a(E203A,E270A) double mutant gave currents that resembled those of the single E203A mutant (Fig. 3, D–F). Like AtClC-a(E203A), the double mutant failed to transport protons in response to depolarization (Fig. 3I).

Two main biophysical differences between AtClC-a and CIC-4 and -5 are (i) the much stronger rectification of the mammalian isoforms and (ii) their partial uncoupling of anion from proton transport in the case of nitrate. We therefore searched for differences in their primary sequences that may underlie these differences. A salient feature is the presence of a proline in these differences. A salient feature is the presence of a proline in the equivalent serine to proline in ClC-5 would change its efficiency of NO$_3^\text{−}$/H$^+$ exchange (data not shown). When the P160S mutation was combined with the uncoupling mutation in the gating glutamate, the resulting double mutant AtClC-a(P160S,E203A) displayed a lower I(NO$_3^\text{−}$)/I(Cl$^\text{−}$) ratio that was similar to the single E203A mutant (Fig. 4F) and had lost proton coupling (data not shown).

The above experiments indicate that the presence of proline in the CLC signature sequence may be responsible for the efficient NO$_3^\text{−}$/H$^+$ coupling of AtClC-a. We asked next whether changing the equivalent serine to proline in CIC-5 would increase its efficiency of NO$_3^\text{−}$/H$^+$ coupling. In the presence of extracellular chloride, currents from the CIC-5(S168P) mutant (Fig. 5D) were much smaller (~5–10-fold) than WT currents (Fig. 5A). They increased ~3-fold in the presence of extracellular NO$_3^\text{−}$ (Fig. 5E), coinciding with the recent parallel work by Zifarelli and Pusch (38). Whereas currents of WT CIC-5 are also larger with NO$_3^\text{−}$ than with Cl$^\text{−}$ (Fig. 5, A–C), the I(NO$_3^\text{−}$)/I(Cl$^\text{−}$) ratio is markedly increased in the mutant (Fig. 5, D–F and K). The strong rectification of CIC-5 currents is not appreciably affected by the S168P mutation (Fig. 5, A–F), but currents showed somewhat more pronounced and slower “gating” relaxations when jumping to positive voltages. In contrast to WT
ClC-5 (Fig. 5G), trains of depolarizing pulses alkalinized the oocyte interior much more rapidly when extracellular Cl⁻/H⁺ was replaced by NO₃⁻/H⁺ (Fig. 5I). ClC-5(S168P) transported H⁺ efficiently against its electrochemical gradient (with pH₅.5) using NO₃⁻/H⁺ as a driving ion (Fig. 5J). When Ser-168 of ClC-5 was replaced by glycine or alanine, only the S168A mutation yielded a moderately higher I(NO₃⁻)/I(Cl⁻) current ratio (Fig. 5K). This contrasts with AtClC-a, where glycine could substitute for proline without compromising its I(NO₃⁻)/I(Cl⁻) ratio (Fig. 4F) and its efficient NO₃⁻/H⁺ exchange. Hence, a single mutation that replaced a serine by proline, which is found at that position in AtClC-a, strongly increased the preference of ClC-5 for NO₃⁻ and converted it into an efficient NO₃⁻/H⁺ exchanger.

The uncoupling E211A mutation in the ClC-5 gating glutamate slightly increased its relative nitrate conductance (Fig. 5K), whereas the equivalent mutation in AtClC-a quite drastically lowered its nitrate versus chloride conductance (Fig. 4F). Exchanging serine 168 for proline in the uncoupled ClC-5 mutant increased relative conductance in the presence of NO₃⁻ (Fig. 5K), whereas a similar exchange in the uncoupled AtClC-a mutant failed to change the I(NO₃⁻)/I(Cl⁻) current ratio (Fig. 4F).

We finally asked whether also CLC Cl⁻/H⁺ channels gain higher NO₃⁻ conductance with an equivalent mutation and used the Torpedo Cl⁻/H⁺ channel ClC-0 (35) as a widely used model channel. When expressed in Xenopus oocytes, the S123P mutant gave robust currents that were, however, ~4-fold smaller than those from WT ClC-0 (Fig. 6, A and D). Whereas the WT channel conducts Cl⁻ much better than nitrate (Fig. 6, A–C), NO₃⁻ conductance was larger than Cl⁻ conductance in the S123P
mutant (Fig. 6, D–F). The selectivity was also changed for other anions, as evident in the large increase of Br^-/H^+ conductance (Fig. 6F). In addition, the mutation drastically slowed the fast protopore gate (as evident from current relaxations after stepping to negative voltages, Fig. 6, A and D) and introduced an open-pore outward rectification (Fig. 6, C and F). The concomitant change of pore and gating properties reflects the tight coupling of permeation and gating in CLC Cl^-/H^+ channels (39).

**DISCUSSION**

We have achieved for the first time the functional expression of a plant CLC transporter in animal cells. This enabled us to study the currents and proton transport of wild-type and mutant AtClC-a, a NO_3^-/H^+ exchanger that serves to accumulate the plant nutrient NO_3^- in vacuoles (16, 37). We have studied the role in AtClC-a of key glutamate residues that are important for anion/proton coupling in bacterial and mammalian CLC isoforms and have shown that a proline-serine exchange in a highly conserved stretch (GSGIPE, the "CLC signature sequence") strongly affects nitrate/proton transport in AtClC-a and ClC-5.

In our previous study, we were unable to detect plasma membrane currents in *Xenopus* oocytes injected with AtClC-a–AtClC-d (12). Currents reported previously for the oocyte-expressed tobacco CLC NtClC (40) resemble endogenous oocyte currents (12). Hence, the present study may represent the first functional characterization of plant CLC proteins in animal cells. This renders AtClC-a accessible to structure-function analysis by mutagenesis. The most important technical differences from our previous study (12) are the injection of about twice the amount of RNA and a longer time of expression. Whereas we previously measured oocytes 3 days after injection, we now found that AtClC-a currents rise above background levels only after 5 days. Because AtClC-a is physiologically expressed in the plant vacuole (16), the presence of plasma membrane currents may indicate a misrouting of AtClC-a in overexpressing oocytes.

The currents reported here for oocyte-expressed AtClC-a resemble in many aspects the currents from *Arabidopsis* vacuoles that were studied by patch clamp in the whole vacuole configuration and that were absent in strains with AtClC-a gene deletions (16). Both currents showed similar rectification and proton coupling. However, there are also conspicuous differences. When studied under asymmetric ionic conditions with NO_3^- in the pipette (vacuole) and Cl^- in the bath (cytosol), which resembles our oocyte experiments with external NO_3^-, vacuolar currents showed prominent, depolarization-induced

**FIGURE 4. A critical role of proline residue in AtClC-a in determining anion selectivity.** A, shown is the sequence alignment of several CLC proteins in the region of the GSGIPE signature sequence. *E. coli* EcClC-1 as well as all animal CLC proteins have a serine between the two glycines. It is replaced by proline in plant AtClC-a and -b, but not in AtClC-c and -d. B and C, shown are typical two-electrode voltage-clamp traces of the AtClC-a P160S mutant in *Xenopus* oocytes with external Cl^- (B) or NO_3^- (C) (voltage protocol as in Fig. 1). D, the current-voltage relationship of the mutant appears to be identical in extracellular Cl^- and NO_3^- (mean of nine oocytes from six batches each). Currents were normalized to those in Cl^- at +80 mV (where mean current was 2.33 ± 0.44 µA). E, depolarization (depol.)-induced H^+ transport of AtClC-a (P160S) is similarly effective with extracellular Cl^- or NO_3^-, in stark contrast to the WT (Fig. 1G,H). Similar results were obtained in 14 experiments (14 oocytes from six batches). F, shown is the ratio of currents at +80 mV in the presence of NO_3^- and Cl^- for AtClC-a and several mutants, respectively. The number of oocytes is as follows: AtClC-a, 25; AtClC-a(E203A), 24; AtClC-a(P160S), 9; AtClC-a(P160G), 5; AtClC-a(P160S,E203A), 4; and AtClC-a(E203A,E270A), 9. rel. fluor., relative fluorescence.
FIGURE 5. Effect of the S168P mutation on ClC-5 conductance and proton coupling. A and B, shown are the typical two-electrode voltage-clamp traces of oocyte-expressed ClC-5 in Cl−-containing (A) or NO3−-containing (B) solutions. C, shown are the steady-state I/V curves from such experiments (averaged from 19 oocytes from seven batches; normalized for each oocyte to current in Cl− at +80 mV, with mean current of 3.01 ± 0.35 μA). D–F, shown are the current properties of the S168P mutant measured as in A–C. The data in F show means from 23 oocytes from five batches, normalized to I in Cl− at +80 mV, with mean I of 0.79 ± 0.17 μA. Voltage-clamp protocols were performed in A–F as described in the legend to Fig. 1. G, shown is an example of less efficient depolarization (depol.-) induced alkalinization by ClC-5 in the presence of NO3− compared with Cl−. Similar results were obtained in 17 experiments. H, hyperpolarization (hyperpol.) does not elicit intracellular acidification with ClC-5, in contrast to AtClC-a (Fig. 1I). Similar results were obtained in 13 experiments. I and J, shown is the drastically increased NO3−/H+ exchange activity with ClC-5 (S168P), as shown by Fluorocyte with pHo 7.5 (I) or with pHo 5.5 (transport against gradient) (J), resembling in this respect AtClC-a (Fig. 1G and H). Similar results were obtained in 21 (I) and eight (J) experiments. K, shown is a ratio of currents at +80 mV measured with extracellular NO3− and Cl−, respectively, for ClC-5 and several mutants, determined as in Fig. 4F. The number of oocytes used for averages are as follows: ClC-5, 19; ClC-S(S168P), 23; ClC-S(S168G), 17; ClC-S(S168A), 2; ClC-S(S168P,E211A), 6; ClC-S(E211A), 10; ClC-S(E211A,E268A), 34). Error bars indicate S.E. rel. fluor., relative fluorescence.
activation that remained incomplete after several seconds (16). Such current relaxations were clearly absent from our recordings. Moreover, whereas our currents increased with extracellular NO₃⁻ only ~1.6-fold, this increase was much more drastic with vacuolar currents (16). Several explanations may be invoked. Current properties may be influenced by different lipid compositions of membranes from oocytes and plant vacuoles, or AtClC-a might endogenously form complexes with other proteins. These other proteins may include structurally unrelated ancillary β-subunits (as known for some mammalian CLC proteins) (10, 41), other AtClC isoforms (12) (CLC proteins function as dimers (22, 25, 30)), or anchoring proteins.

The outward rectification of AtClC-a is less strong than that of ClC-4 and -5, both of which do not transport measurably at negative voltages (26). This difference is not due to the presence of proline instead of serine in the signature sequence of AtClC-a, as revealed by our mutagenesis experiments. The weaker rectification of AtClC-a may be crucial for the proton-glutamate exchange (15). Unlike equivalent mutations in ClC-4 and ClC-5, this mutation drastically changed current rectification, with both inward and outward rectification being observed. Unlike equivalent mutations in ClC-4 and -5, this uncoupling AtClC-a mutation also drastically changed the I(NO₃⁻). I(Cl⁻) current ratio. This change did not depend on the presence of a proline in the signature sequence.

In CLC anion/proton exchangers, the pathways of Cl⁻ and H⁺ diverge at a point approximately half-way through the membrane and reach the cytoplasm at different points (27). A proton glutamate at the cytoplasmic CLC surface is thought to bind protons, handing them over to the gating glutamate using a poorly defined path. In both mammalian and E. coli CLC exchangers, this glutamate could be replaced by other titratable amino acids without abolishing anion/proton coupling (21, 27, 42). When mutated to nontitratable residues, however, Cl⁻ and H⁺ transport were below detection levels in ClC-4 and -5 (21), whereas small, uncoupled anion currents were observed with EcClC-1 (42). This can be rationalized by a blockade of anion/
Nitrate/Proton Coupling in CLC Transporters

proton exchange at the gating glutamate, when the supply of protons ceases (21). The uncoupled but reduced anion flux in EcClC-1 might be owed to “slippage” past the central exchange site (42). This model is strongly supported by double mutants in CIC-4 and -5, in which the uncoupling gating glutamate mutation rescued the uncoupled anion flux (21). Exactly this situation was found here with AtCIC-a.

It was puzzling that anion transport of CIC-4, -5, and EcClC-1 is largely uncoupled from proton transport with the polyatomic anion NO$_3^-$ (21), whereas AtCIC-a efficiently couples NO$_3^-$ to proton countertransport (16), an essential property for its role in accumulating this plant nutrient in vacuoles. Using sequence comparison, we identified a proline in the CLC signature sequence as a likely candidate for this difference. In all animal CLC proteins, this position is occupied by serine. Indeed, when Pro-160 in AtClC-a was mutated to serine, the plant transporter performed the Cl$^-$/H$^+$ exchange with similar efficiency as the NO$_3^-$/H$^+$ exchange, rather than preferring NO$_3^-$ as in the WT. Importantly, when the equivalent serine of CIC-5 was mutated to proline, CIC-5 mediated an efficient NO$_3^-$/H$^+$ exchange both in the present study as well as in parallel work by Zifarelli and Pusch (38). A novel method (43) to measure proton transport allowed these authors to show that CIC-5(S168P) had gained an NO$_3^-$/H$^+$ coupling ratio of $\sim$2 at voltages between +40 and +60 mV, indistinguishable from that for Cl$^-$/H$^+$ exchange (38). Our data on other CIC-5 mutants at this position (Fig. 5K) largely agree with their results (38) but show interesting differences with data obtained for AtCIC-a (Fig. 4F). The substitution of the critical proline by glycine in AtCIC-a did not interfere with its efficient NO$_3^-$/H$^+$ exchange, possibly suggesting that a helix breaker might be sufficient to support such an exchange. However, glycine at the equivalent position in CIC-5 does not increase currents in the presence of NO$_3^-$ nor does it enable efficient NO$_3^-$/H$^+$ exchange (data not shown) (38).

Only four of seven Arabidopsis CLC proteins have a proline in their signature sequence, with the remaining three displaying a serine like all known animal CLC proteins. This suggests that all these four proline-containing AtCIC proteins function as 2NO$_3^-$/H$^+$ exchangers. A more definitive assignment of their physiological roles, however, is not yet possible. In this respect, it is interesting to note that only AtCIC-c and AtCIC-d were reported to complement (12, 32) growth phenotypes of a Saccharomyces cerevisiae strain deleted for the single yeast CLC (ScCIC or Gef) (33). Whether this is related to the fact that AtCIC-c and -d, just like ScCIC, carry serine in their signature sequence and hence probably prefer chloride over nitrate remains unclear.

The interpretation of conductance ratios of CIC-5, and by extension of AtCIC-a, is complicated by results from a noise analysis that indicates that CIC-5 switches from transporting to nontransporting modes of operation (21). Such a gating of transport activity probably underlies the time-dependent current relaxation upon depolarization of CIC-5 and might also explain the slow depolarization-induced activation of vacuolar currents observed by De Angeli et al. (16). Zifarelli and Pusch (38) recently concluded from their noise analysis that the increase in CIC-5 currents with NO$_3^-$ is not due to an increased “unitary conductance” of the “turned on” transporter (which would include slippage of the anion) but rather to a higher “open probability” of the transporter. However, it seems unlikely that the difference in $I$(NO$_3^-$)/$I$(Cl$^-$) ratios between WT AtCIC-a and mutant P160S is solely due to an effect on gating. This is because this mutation not only modified conductance ratios but also changed the apparent permeability (reversal potentials). We therefore conclude that the proline-serine exchange affects the ion selectivity of the exchange process. This conclusion is indirectly bolstered by the single-channel analysis of the CIC-0(S123T) mutant, which demonstrated changes in ion selectivity and other pore properties (25) and by the changed ion selectivity of the CIC-0(S123P) mutant described here. Unfortunately, we cannot draw similar conclusions for CIC-5 because its strong rectification precludes the determination of reversal potentials.

In the crystal of EcClC-1, the equivalent serine participates in the coordination of a Br$^-$ ion (used as a Cl$^-$ substitute in crystallography) in the central binding site (22). Several mutations in Tyr-445, another residue involved in this coordination (22), uncoupled chloride from proton fluxes (44). Such mutations were associated with a reduced presence or complete absence of anions at the central binding site (44). Likewise, crystals obtained in the presence of NO$_3^-$ revealed that this anion, which uncouples Cl$^-$ from H$^+$ transport in EcClC-1, cannot be detected at this position (18). Thus, anion/proton coupling seems to require that an anion occupies this site. It was proposed that this anion serves as an intermediate binding site for protons on their way from the proton glutamate to the gating glutamate, leading to the seemingly outlandish proposal of HCl as a proton transport intermediate (45). We suggest that the replacement of serine by proline in the GSGIPE sequence enables NO$_3^-$ to occupy the central anion binding site, thereby leading to efficient proton coupling.

In summary, a breakthrough in heterologous expression of AtCIC-a has allowed us to extend the structure-function analysis of anion/proton exchange to plant CLC proteins. Mutagenesis of critical gating and proton glutamates resulted in changes of proton coupling and rectification that bear close resemblance to results from mammalian endosomal CLC proteins. However, there were also significant differences in effects on NO$_3^-$ conductance. We further identified an important proline residue in the CLC signature sequence of AtCIC-a that is crucial for its efficient NO$_3^-$/H$^+$ exchange activity and that conferred more efficient NO$_3^-$/H$^+$ coupling on the mammalian CIC-5 exchanger and an increase in NO$_3^-$ conductance on the Torpedo Cl$^-$ channel CIC-0. Our work provides a basis for future studies of other plant CLC proteins and their comparison to mammalian counterparts.

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Nitrato/Proton Coupling in CLC Transporters

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