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Mutational Analysis Demonstrates That ClC-4 and ClC-5 Directly Mediate Plasma Membrane Currents*

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ClC-4 and ClC-5, together with ClC-3, form a distinct branch of the CLC chloride channel family. Although ClC-5 was shown to be mainly expressed in endocytotic vesicles, expression of ClC-5 in *Xenopus* oocytes elicited chloride currents. We now show that ClC-4 also gives rise to strongly outwardly rectifying anion currents when expressed in oocytes. They closely resemble ClC-5 currents with which they share a $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence that differs from that reported for the highly homologous ClC-3. Both ClC-4 and ClC-5 currents are reduced by lowering extracellular pH. We could measure similar currents after expressing either channel in HEK293 cells. To demonstrate that these currents are directly mediated by the channel proteins, we introduced several point mutations that change channel characteristics. In ClC-5, several point mutations alter the kinetics of activation but leave macroscopic rectification and ion selectivity unchanged. A mutation (N565K) equivalent to a mutation reported to have profound effects on ClC-3 does not have similar effects on ClC-5. Moreover, a mutation at the end of D2 (S168T in ClC-5) changes ion selectivity, and a mutation at the end of D3 (E211A in ClC-5 and E224A in ClC-4) changes voltage dependence and ion selectivity. This shows that ClC-4 and ClC-5 can directly mediate plasma membrane currents.

The CLC[1] family of chloride channels, originally defined by the ClC-0 chloride channel from *Torpedo* electric organ (1), comprises nine known members in mammals (2). Mutations in three of the corresponding genes are known to cause human disease: mutations in ClC-1 cause myotonia (3, 4), mutations in ClC-Kb Bartter's syndrome (5), and mutations in ClC-5 cause Dent's disease (6).

Dent's disease is an X chromosome-linked disorder and has two main symptoms: hypercalciuria, which leads to kidney stones, nephrocalcinosis, and renal failure, and second, low molecular weight proteinuria (7). The proteinuria points to a defect in endocytosis of proximal tubular cells. Indeed, ClC-5 is expressed in the proximal tubule and in intercalated cells of the distal nephron (8). Both cell types are involved in endocytosis. The localization of ClC-5 in intracellular vesicles and its colocalization with the proton pump suggests that it provides an electrical shunt necessary for an efficient acidification of these vesicles. This defect in intravesicular acidification probably leads to the impaired endocytosis of proteins observed in Dent's disease. In transfected cells, ClC-5 was present in intracellular vesicles. In addition, there was also some labeling of the plasma membrane (8).

Consistent with a plasma membrane localization, expression of ClC-5 in *Xenopus* oocytes elicited chloride currents (9). These had a $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence and were strongly outwardly rectifying. Currents were detectable only at voltages more positive than +20 mV. When we analyzed ClC-5 mutations found in Dent's disease (6, 10–12), currents were either significantly decreased or abolished. In contrast to ClC-1 mutations in myotonia (13), we never found changes in rectification, voltage dependence, or ion selectivity. Thus, we could not rule out the possibility that these currents were mediated by a different channel that was activated by expressing ClC-5. Mutations compromising ClC-5 function would then change those currents quantitatively without changing their characteristics. Given that ClC-5 plays a role in endocytosis (8), ClC-5 expression may change the plasma membrane localization of channels that are endogenous to the expression system. Thus, identifying mutations that change current characteristics seemed of high priority.

Despite these open questions, ClC-5 is the best understood member of the ClC-3/4/5 branch of the *CLC* gene family. These three proteins are about 80% identical (9). In previous studies, we did not observe currents upon ClC-4 expression (14). While some groups reported that ClC-3 expression gave no currents (14, 15), others reported outwardly rectifying currents with an $I^- > Cl^-$ selectivity (16–19). The work of Duan *et al.* (18) suggests that ClC-3 represents the ubiquitous swelling-activated chloride channel. These authors introduced a mutation into ClC-3 that was equivalent to a mutation changing pore properties in other CLC channels (20–22) and found the expected changes in ClC-3-induced currents (18).

The present study has two major aims. First, to compare several electrophysiological properties of currents elicited by these highly homologous proteins. Second, to prove that these currents are directly due to these gene products by introducing mutations that change their properties. For the first time, ClC-4 gave chloride currents when expressed in *Xenopus* oocytes or HEK293 cells. These resemble ClC-5 currents in many respects, but differ slightly in voltage dependence and pH sensitivity. Using various point mutations we demonstrate that these currents are indeed mediated by ClC-4 and ClC-5. Surprisingly, their properties differ significantly from those reported for ClC-3 (16–19), and we could not detect currents upon ClC-3 expression.

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[1] The abbreviations used are: CLC, chloride channel of the *CLC* gene family; ClC-X, member X of the *CLC* gene family; HEK, human embryonal kidney; GFP, green fluorescent protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; NMDG, *N*-methyl-D-glucamine; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; WT, wild-type.

EXPERIMENTAL PROCEDURES

ClC-4 and ClC-5 Constructs—A human ClC-4 cDNA was cloned into pTLN (23) or pCIneo (Promega, Madison, WI). It differed from the published sequence (24) (GenBank™ accession number X77197) at the following residues: Ala instead of Arg (GenBank™) at 178; Ile-Ile instead of Tyr-Tyr at 498 and 499; and Lys instead of Asn at 659. The residues we found are also present in the rat (GenBank™ accession number Z36944) and mouse (GenBank™ accession number S47327) cDNAs. The 5′ end of ClC-4 contains three ATGs in frame (at amino acid numbers 1, 7, and 14; the last one corresponds to the ClC-5 initiator ATG). We could not detect functional differences between these and therefore used the first ATG for all subsequent studies. Amino acids are numbered starting from this methionine. Mutations were introduced by recombinant polymerase chain reaction. All polymerase chain reaction-derived fragments were fully sequenced. The cDNA of ClC-5 contained the rat sequence between the initiator ATG and the *Dra*III restriction site, which is 100% identical to the human sequence on the protein level.

Expression in Xenopus laevis Oocytes and Voltage-Clamp Studies—Using SP6 RNA polymerase capped cRNA was transcribed from the constructs after linearization. 10–25 ng of cRNA were injected into *Xenopus* oocytes isolated by manual defolliculation as described (1). Oocytes were kept at 17 °C in modified Barth's solution (88 mM NaCl, 1.0 mM KCl, 1.0 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, pH 7.6). Two-electrode voltage-clamp measurements were performed at room temperature 2–3 days after injection using Turbotec 05 or 10C amplifiers (NPI Instruments, Tamm, Germany) and pClamp 5.5 software (Axon Instruments). Currents were recorded in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Na-HEPES, pH 7.4). For anion replacement, 80 mM Cl^- was substituted by equivalent amounts of Br^-, I^-, NO_3^-, or glutamate. When using different pH values, 5 mM HEPES (for pH 7.4) were replaced by 5 mM MES (for pH 6.5 and 5.5) or 5 mM TAPS (for pH 8.5). Permeability coefficients were calculated from reversal potential measurements under biionic conditions using the Goldman-Hodgkin-Katz equation (25).

Whole Cell Patch-Clamp Measurements—HEK293 cells were transiently transfected with ClC-4 or ClC-5 cDNA (WT or mutants, subcloned into pCIneo) using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer's procedures. To identify transfectants, a green fluorescent protein construct (pEGFP; CLONTECH) was cotransfected. Whole cell patch-clamp measurements were performed 30–72 h after transfection at room temperature in an extracellular solution containing 140 mM NMDG, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.4, using an Axopatch 200A amplifier (Axon Instruments) and pClamp5.5 software. Patch pipettes were pulled from borosilicate glass to 2–5 μm tip diameter and filled with either high-Cl intracellular solution (140 mM NMDG, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM HEPES, pH 7.4) or low-Cl intracellular solution (120 mM NMDG-aspartate, 20 mM NMDG-Cl, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM HEPES pH 7.4). Pipette resistances were in the range of 1–5 MΩ. Data were usually digitized at 2 kHz frequency after filtering at 1 kHz.

RESULTS

To reinvestigate the functional expression of ClC-4, we cloned the human ClC-4 cDNA into an optimized expression vector (23) and injected derived cRNA into *Xenopus* oocytes. After 2–3 days, two-electrode voltage-clamping revealed strongly outwardly rectifying chloride currents (Fig. 1*a*) that were absent from control oocytes. Currents activated rapidly at positive voltages and in many respects resemble ClC-5 currents (9). No tail currents could be detected when stepping back to negative voltages. Similar currents were observed when ClC-4 or ClC-5 were studied in transfected HEK293 cells (Fig. 1, *c* and *d*, respectively). This excludes that these currents are due to the activation of a chloride channel specific for *Xenopus* oocytes. Since the intracellular solution is buffered with EGTA, both channels do not depend on intracellular calcium. In contrast to ClC-5, where currents begin to activate at voltages more positive than +20 mV ((9) and Fig. 1*e*), ClC-4-induced currents are already visible at slightly more negative potentials and depend less steeply on voltage (Fig. 1*e*). Partial replacement of extracellular chloride by other anions indicated a $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence (Fig. 1*b*). This is again similar to ClC-5 (9) and readily distinguishes these currents from outwardly rectifying, endogenous oocyte currents that display an $I^- > Cl^-$ conductance (9). It is not possible to determine permeability ratios as both channels do not mediate large enough currents at the chloride equilibrium potential.

Currents mediated by either ClC-4 or ClC-5 are markedly reduced by extracellular acidification (Fig. 2). A similar pH sensitivity was recently described for a *Xenopus* CLC channel that may be the species homolog of ClC-5 (26). ClC-5 currents respond to pH changes already in the neutral range and are reduced to less than 50% at pH 5.5 (Fig. 2*a*). By contrast, ClC-4 currents are nearly unaffected in the neutral pH range, and currents begin to decrease when extracellular pH drops below pH 6.5 (Fig. 2*b*). We could not measure currents at more acidic pH reliably because currents in *Xenopus* oocytes became unstable. Nonetheless, our results suggest that the pH dependence of ClC-4 is shifted toward more acidic pH values when compared with ClC-5 (Fig. 2*c*).

Although the rather subtle differences between the currents induced by ClC-4 and ClC-5 suggest that they are directly mediated by these proteins, we sought more definitive evidence from mutational analysis. Mutations associated with Dent's disease either abolished or reduced ClC-5 currents without changing their biophysical properties (6, 10–12). S244L and S520P are mutations that resulted in reduced but otherwise typical currents (6). The mutated amino acids are located in putative transmembrane domains D5 and D11, respectively (Fig. 3*f*). We mutated these critical residues to other amino acids (alanine and threonine). While S244A currents were similar to WT (Fig. 3*a*), the other mutations significantly slowed the activation of ClC-5 (Fig. 3, *b*, *c*, and *d*). This was observed both in transfected HEK293 cells (Fig. 3) and in *Xenopus* oocytes (not shown). However, we did not find changes in ion selectivity or in steady-state rectification. As with WT channels, we could not use tail currents to measure the rectification of the open pore.

Residues affecting ion selectivity and rectification were found in several regions of CLC channels (27). The first mutation shown to affect pore properties was K519Q (and K519E) in ClC-0 (20). This lysine at the end of a large transmembrane block is conserved within ClC-0, -1, -2, and the two ClC-K isoforms. Both mutations reduced the $Cl^- > I^-$ selectivity of ClC-0, introduced an outward rectification, slowed gating, and reduced its single channel conductance (20, 27, 28). In ClC-3, -4, and -5, an asparagine is present at the equivalent position. We changed this asparagine to a lysine in ClC-5 (N565K). Its effect may be opposite to that of K519Q, particularly since the effect of that ClC-0 mutation may be due to the charge of the side chain (28). This would predict a more linear I − V relationship and an increase in the Cl^-/I^- conductance ratio. However, this is not the case. While the N565K mutation slightly slowed the activation of ClC-5 currents (Fig. 3*e*), it did not change the overall rectification (Figs. 3*e* and 4*a*). It slightly increased the relative iodide conductance (Fig. 4*a*). Thus, it is not a mirror image of the *Torpedo* K519Q mutation. Nevertheless, it shows that these currents are directly mediated by ClC-5 and that residues at the end of D12 influence pore properties.

Several other regions modulate CLC pore properties. In ClC-0, a conservative mutation (S123T) in a highly conserved stretch (GSGIPE) at the end of D2 increases bromide and iodide conductances relative to chloride (27). The equivalent mutation (S168T) also reduces the ion selectivity of ClC-5 (Fig. 4*b*). At positive voltages, bromide and iodide conductances are increased with respect to chloride, an effect that is more drastic than that of the ClC-0 S123T mutation (27).

Several studies (19, 29–31) reported that mutations in an-

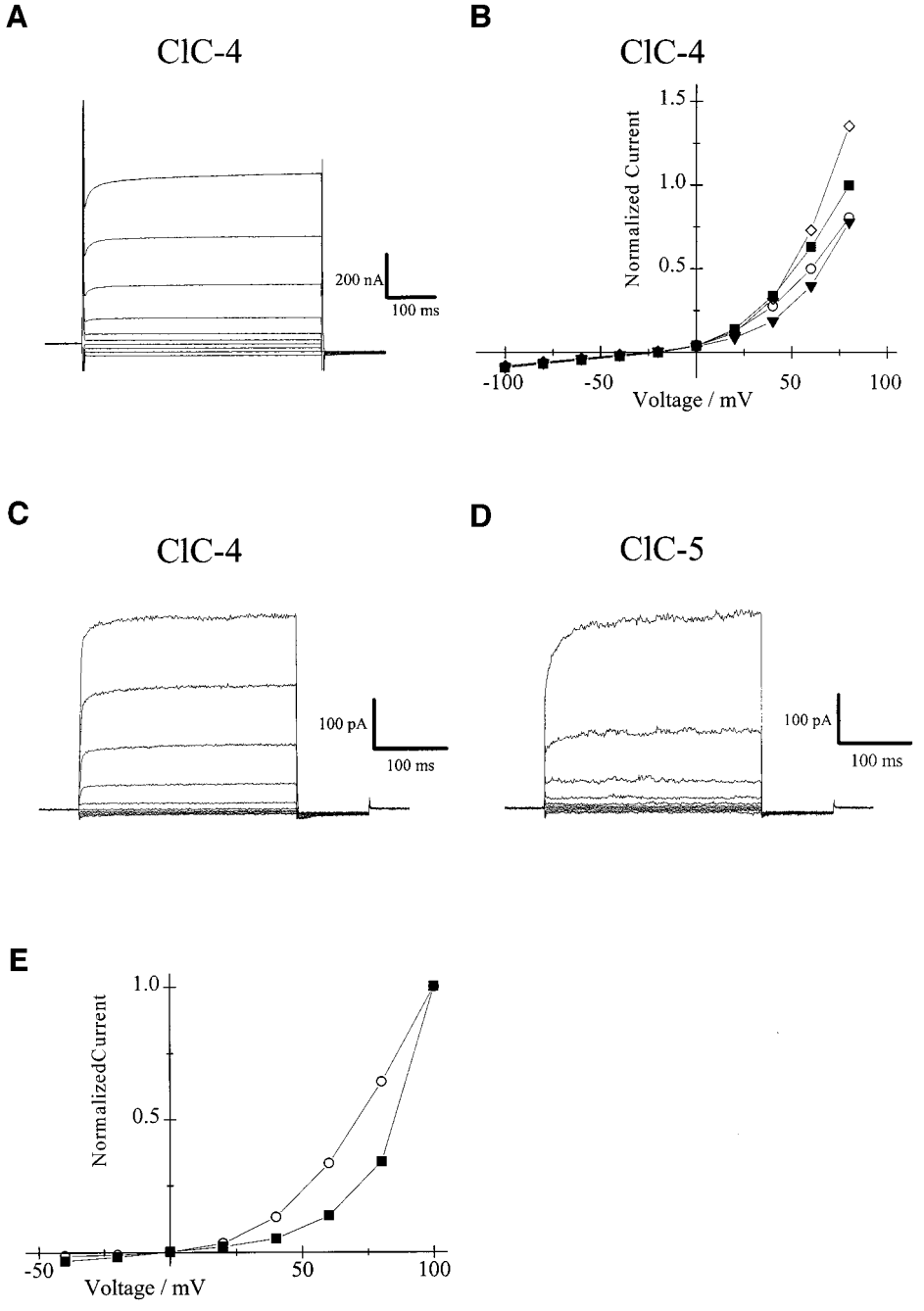

FIG. 1. **Basic electrophysiological properties of human ClC-4 and ClC-5.** *a*, voltage-clamp traces of ClC-4 expressed in *Xenopus* oocytes. Currents were measured in ND96. From a holding potential of −35 mV, the oocyte was clamped in 20-mV steps to voltages between +80 and −100 mV, followed by a constant test pulse at −80 mV. *b*, ion selectivity of ClC-4 expressed in *Xenopus* oocytes and measured as in *a*. 80 mM extracellular chloride was substituted by different anions. (■, ND96; ▼, 80 mM I^-; ○, 80 mM Br^-; ◇, 80 mM NO_3^-). Data were averaged from 29 oocytes from 4 different batches (standard errors were smaller than symbol size). Before averaging, currents of individual oocytes were normalized to the current at +80 mV in ND96 solution. *c* and *d*, whole-cell patch-clamp traces of HEK293 cells transiently transfected with ClC-4 (*c*) or ClC-5 (*d*). Patch pipettes contained the high chloride intracellular solution, and cells were bathed in the extracellular solution. From a holding potential of −30 mV, cells were clamped to voltages between +100 and −100 mV in 20-mV steps, followed by a constant pulse to −60 mV. *e*, steady-state I − V relationships of ClC-4 (○) and ClC-5 (■) currents from whole-cell experiments in *c* and *d*. Amplitudes were taken from currents at the end of the test pulse and normalized for each experiment to the value at +80 mV to allow a direct comparison of channel rectification.

other highly conserved region, GKEGP at the end of D3, also have effects on gating and pore properties. We mutated the glutamate to alanine in ClC-4 (E224A) and in ClC-5 (E211A). There was a drastic effect on gating (Fig. 4, *c* and *d*). Both channels now mediate significant currents in the negative voltage range. We could not detect significant current relaxations, suggesting that gating is now either substantially faster or does no longer depend on voltage. There was also a slight increase in bromide conductance at positive voltages, the only range where a comparison to WT channels is possible (Fig. 4, *e* and *f*). The changed voltage dependence allowed us to measure permeability ratios that indicated a NO_3^- (1.5) = Br^- (1.45) > Cl^- (1.0) = I^- ratio for ClC-4(E224A), and a Br^- (1.2) > Cl^- (1.0) = NO_3^- > I^- (0.7) sequence for ClC-5(E211A). It is unclear how these differ from the WT permeabilities which cannot be measured.

Given the strong similarity between ClC-4 and ClC-5 currents, it is surprising that ClC-3 was reported to elicit less

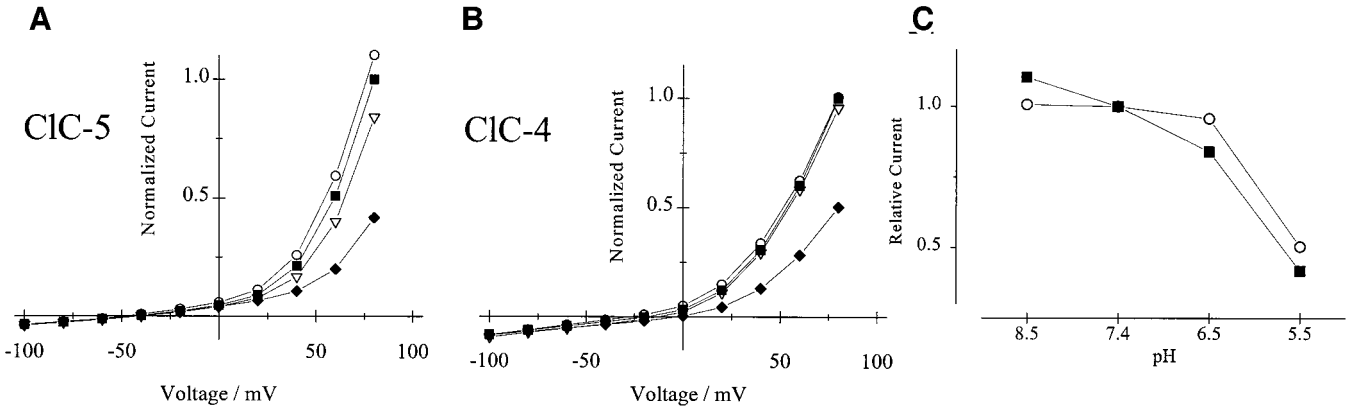

FIG. 2. **pH dependence of ClC-4 and ClC-5 currents.** Steady-state I − V curves of ClC-5 (*a*) and ClC-4 (*b*) expressed in *Xenopus* oocytes. Extracellular pH was varied in four steps between pH 8.5 and 5.5, and currents were measured as in Fig. 1*a*. Currents were normalized for individual oocytes to the current at +80 mV in ND96 (pH 7.4) before averaging. Data are from 36 oocytes (from 3 different batches) for *a* and from 27 oocytes (from 4 different batches) for *b*. Symbols for the different pH values are as follows. ○, pH 8.5; ■, pH 7.4; ▽, pH 6.5; ◆, pH 5.5. Standard errors were smaller than symbol size and therefore not shown. *c*, pH dependence of ClC-4 (○) and ClC-5 (■) currents measured at +80 mV from the same set of data as in *a* and *b*. Current amplitudes were normalized to the value at pH 7.4.

strongly rectifying channels with an $I^- > Cl^-$ selectivity (16–19). In previous experiments, we (9, 14) and others (15) were unable to obtain ClC-3 currents in *Xenopus* oocytes. We have now repeated these experiments with human and guinea pig (18) ClC-3 cDNAs cloned into the expression vectors used in this study. We again failed to observe currents with human ClC-3 in *Xenopus* oocytes. Attempts to express chloride currents with the guinea pig ClC-3 in transiently transfected HEK293 or NIH3T3 cells were unsuccessful as well.

DISCUSSION

ClC-4 and ClC-5, together with ClC-3, form a distinct branch of the *CLC* gene family (2). This branch has received much attention because ClC-5 mutations cause Dent's disease (6) and because ClC-3 was recently proposed (18) to represent the long sought swelling-activated chloride channel. ClC-4 did not elicit currents in previous studies, and attempts to express ClC-3 gave conflicting results (9, 15–19). Furthermore, a formal proof that the strongly outwardly rectifying currents provoked by ClC-5 are directly mediated by this channel was missing.

We now demonstrate that both ClC-4 and ClC-5 directly mediate plasma membrane currents that are very similar. This includes their extreme outward rectification, the lack of significant tail currents, and the $NO_3^- > Cl^- > Br^- > I^-$ conductance sequence at strongly depolarizing potentials. Both ClC-4 and ClC-5 currents were sensitive to extracellular pH. However, they are not identical. Given these differences, it is unlikely that both proteins activate an identical endogenous oocyte channel, an otherwise frequently observed phenomenon with *Xenopus* oocytes (32). This is also unlikely in view of the very similar currents in different expression systems (*Xenopus* oocytes and HEK293 cells).

The observed pH dependence of ClC-4 and ClC-5 possibly points to an important physiological role of these channels. ClC-5 is expressed in the endocytotic pathway where it may provide the electrical shunt necessary for an efficient acidification of the vesicle interior (8). A similar role in intracellular organelles was proposed for the yeast CLC channel (33–35). Topologically, the vesicle interior corresponds to the extracellular space. An inhibition of chloride currents by acidic intravesicular pH will provide a negative feedback on proton pumping and could be important for setting the pH of these vesicles. Along the endocytotic pathway, the intravesicular pH gets progressively more acidic from early (pH 6.0–6.5) to late (pH 5.0–6.0) compartments (36). The pH dependence of ClC-5 fits well with a localization in the early endocytotic pathway (8). An assignment for the physiological role of the pH dependence of ClC-4 is not yet possible and requires an exact localization of that channel.

It is puzzling that both channels yield significant currents only at very positive voltages. The voltage across intracellular vesicles is not well known, but the electrogenic proton pump is expected to create an inside positive voltage. Topologically this corresponds to a negative voltage in our measurements, which would prevent a significant opening of ClC-4 or ClC-5. However, we cannot exclude small currents due to non-zero open probability at negative voltages. Transport rates of active pumps are orders of magnitudes lower than those of ion channels. Hence, even such small channel-mediated currents may suffice to allow efficient proton pumping. Alternatively, there may be other subunits that alter the voltage dependence. Some CLC proteins can form heteromeric channels with novel properties (23), but we observed no qualitative changes when co-expressing ClC-5 with either ClC-4 or ClC-3 (not shown). CLC channels may also have as yet unknown β-type subunits.

The most compelling evidence for a direct channel function of ClC-4 and ClC-5 comes from our mutagenesis experiments. Several conservative amino acid exchanges slowed the activation of ClC-5 currents in both expression systems. However, changing gating kinetics by mutagenesis does not prove that these proteins are directly ion channels. Indeed, gating kinetics is often drastically affected by β-subunits that do not form the pore (37) and may even be changed by expression levels (38). More convincing than mutations changing kinetics are those that change pore properties such as ion selectivity and/or rectification.

The pore of CLC channels has not yet been defined. Point mutations at different positions change pore properties in ClC-0, -1, and -2. This includes residues at the ends of transmembrane domains D2 (27), D3 (19, 29–31), and D12 (20–22, 27, 28, 39). Mutations in all three regions also changed properties of ClC-5. A mutation at the end of D12 (ClC-5(N565K)) slightly altered both gating kinetics and conductance ratios. The most drastic effect on conductance ratios was exerted by a mutation in the highly conserved GSGIPE region at the end of D2. A mutation at the end of D3 (ClC-4(E224A) and ClC-5(E211A)) had drastic effects on rectification of both channels and also slightly changed their conductance sequences. Several mutations in the same region in ClC-1 yielded channels with large inward currents (19), as did a mutation in the correspond-

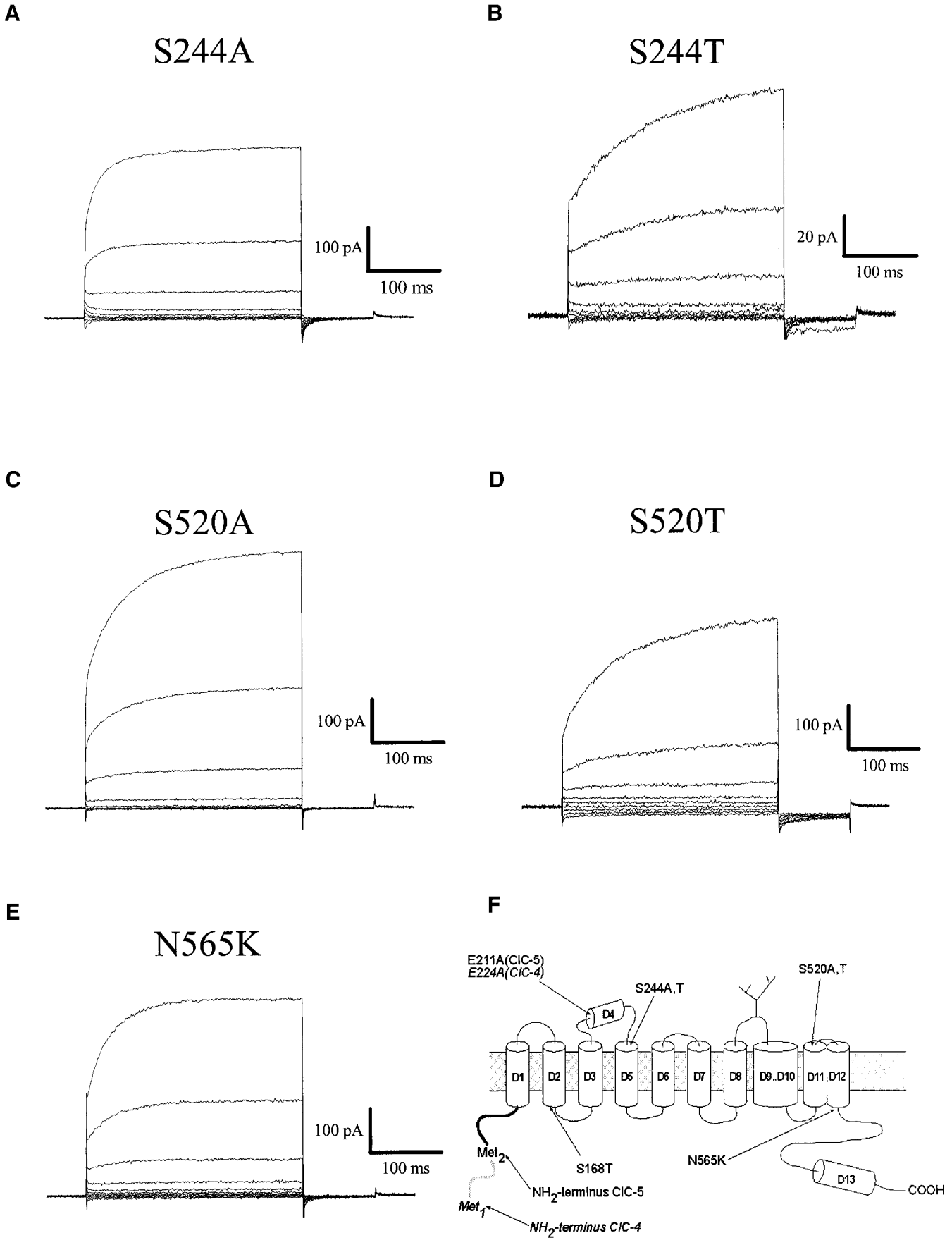

FIG. 3. **Mutants of ClC-5 displaying slower activation kinetics than WT.** HEK293 cells were transfected with the S244A (*a*), S244T (*b*), S520A (*c*), S520T (*d*), and N565K (*e*) mutants of ClC-5 and examined by whole-cell patch-clamp experiments. For comparison with WT currents, see Fig. 1*d*. Measurements were done as in Fig. 1*d* with low Cl^- intracellular solution, but the voltage range was between +120 mV and −100 mV. Time constants for current activation were determined to: WT, τ = 32 ± 3 ms; S244A, 31 ± 2 ms; S244T, 74 ± 3 ms; S520A, 58 ± 2 ms; S520T,

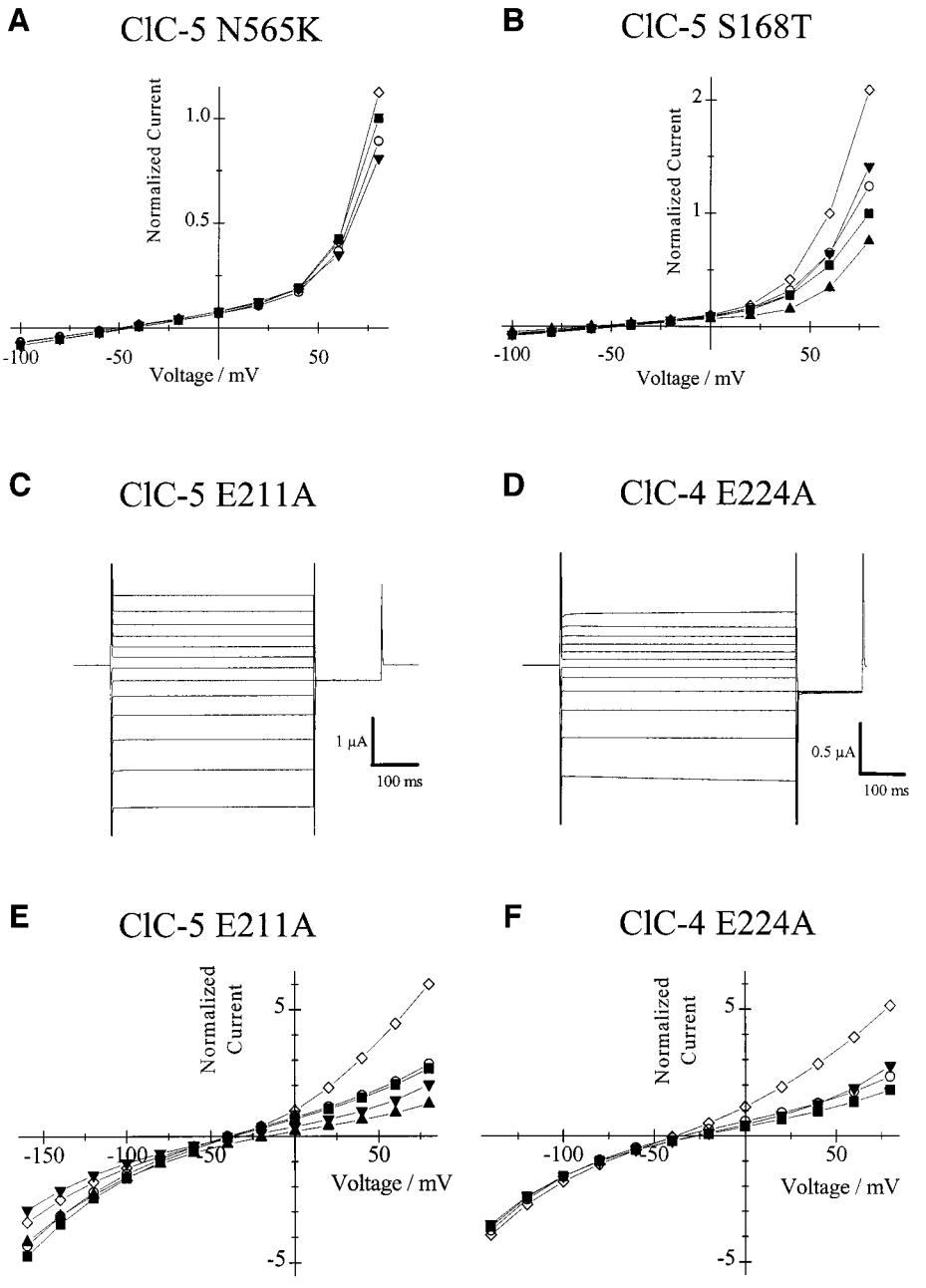

FIG. 4. **Mutants of hClC-4 and hClC-5 with changed ion selectivity and rectification**. Mutant channels were expressed in *Xenopus* oocytes and examined by two-electrode voltage clamping as described in Fig. 1*a*. *a*, I − V relationship of ClC-5(N565K) in the presence of different extracellular anions. *b*, I − V curves of the ClC-5 S168T mutant with different extracellular anions. Currents were normalized to the current at +80 mV in ND96 (average from 31 oocytes from of 3 different batches). *Bars* indicate standard errors. Solutions used for anion exchange experiments and symbols are as follows. Starting from ND96 (■), 80 mM Cl^- were replaced by 80 mM Br^- (○), 80 mM I^- (▼), 80 mM NO_3^- (◇), or 80 mM glutamate (▲) for all panels of the figure. *c*, voltage-clamp traces of ClC-5(E211A); *d*, traces from the equivalent ClC-4 mutant E224A. Oocytes were clamped in 20-mV steps between +80 and −160 mV (*c*) and +80 and −140 mV (*d*) from a holding potential of −35 mV. *e* and *f* display the corresponding I − V curves measured in the presence of different extracellular anions for ClC-5(E211A) and ClC-4(E224A), respectively. These were obtained using pulse protocols as in *c* and *d*, respectively. Currents were normalized to the current at −80 mV in ND96 solution for individual oocytes. Data were averaged from 18 oocytes (from 3 different batches) for *e* and *f*. Standard errors were smaller than symbol size.

ing region of ClC-0 (40). Many mutations altering the ion selectivity also changed the kinetics or the voltage dependence of gating or both. This has been observed previously also with ClC-0 and ClC-1 (19–21, 27). Indeed, permeation and gating is intimately linked in CLC channels (20, 21, 41, 42). In contrast to ClC-0 (20) and ClC-1 (21), however, anion substitution did not appreciably change the voltage dependence of ClC-4 or ClC-5 (Fig. 1*b* and Ref. 9). This is compatible with the notion that the conductance ratios of our measurements reflect pore properties and are not just a consequence of changes in open probability.

The $Cl^- > I^-$ selectivity sequence of ClC-4 and ClC-5 agrees well with that of ClC-0 (20), ClC-1 (19, 29), and ClC-2 (22, 43, 44) and may be a general property of CLC channels. Our data on ClC-5 (9) differ from those of Sakamoto *et al.* (45). They proposed an $I^- > Cl^-$ selectivity, although the shift of reversal potentials upon ion substitutions (45) suggests a $Cl^- > I^-$ selectivity. Their currents are less rectifying and sensitive to the chloride channel inhibitor DIDS, which was ineffective in our study (9). *Xenopus* ClC-5 also elicited less rectifying, DIDS-sensitive currents with an $I^- > Cl^-$ selectivity (46). However, when expressed from vectors optimizing expression in oocytes, the same authors reported currents similar to those obtained in our laboratory, and the previously described currents (46) were probably endogenous to oocytes[2] (26). Indeed, injection into *Xenopus* oocytes of cRNAs encoding several unrelated proteins

[2] N. Wills, personal communication.

78 ± 4 ms; N565K, 42 ± 2 ms (values are means ± S.E.; $n = 5$). *f*, topology model for CLC channels showing the position of amino acids mutated in this work. Note that ClC-4 has a longer amino terminus than ClC-5, leading to different numbering of equivalent positions. Terminology of domains D1 through D13 is based on Ref. 1, and topology is drawn according to the experimental results of Ref. 30. However, there are conflicting results (19) for the D4-D5 region.

can elicit similar outwardly rectifying chloride currents with an $I^- > Cl^-$ selectivity (41).

Currents mediated by ClC-4 and ClC-5 are very similar but differ significantly from those reported for ClC-3 (16–19). This is surprising because these proteins are about 80% identical, and ClC-3 is even slightly more homologous to ClC-4 than is ClC-5. Expression of ClC-3 in *Xenopus* oocytes (16) or transfected mammalian cells (17–19) was associated with outwardly rectifying currents with an $I^- > Cl^-$ selectivity. However, the channels reported by Duan *et al*. (18) and Kawasaki *et al*. (17) differ in single channel conductance, rectification, and calcium sensitivity. Duan *et al*. (18) suggest that ClC-3 represents the important swelling-activated chloride channel. This was supported by a mutation introducing a positive charge at the end of D12 (N579K). The current-voltage relationship apparently changed from outwardly rectifying to linear and ion selectivity from $I^- > Cl^-$ to $Cl^- > I^-$ (18). This was expected since a mutation in ClC-0 (K519Q), which neutralizes a charge at this position, has roughly the opposite effect on selectivity and rectification (20). Surprisingly, the equivalent mutation (N565K) in the highly homologous ClC-5 channel, which has a $Cl^- > I^-$ selectivity to begin with, rather slightly increases iodide conductance relative to chloride and has no effect on rectification (Fig. 4*a*). Moreover, we were again unable to measure ClC-3 currents in oocytes and in transfected cells. We have no explanation for these discrepancies.

In view of the predominantly intracellular localization of ClC-5 (8), future studies should address the subcellular distribution of the highly homologous ClC-3 and ClC-4 channels. Preliminary experiments show that ClC-4 is also predominantly present in intracellular vesicles.[3] It will be interesting to see whether the (partial) surface localization, as demonstrated here for ClC-4 and ClC-5, serves a physiological role or is the by-product of a recycling via the plasma membrane. In any case, the surface expression allows a biophysical analysis of channels whose primary function may be in intracellular acidification.

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