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

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CIC-4 and CIC-5, together with CIC-3, form a distinct branch of the CLC chloride channel family. Although CIC-5 was shown to be mainly expressed in endocytotic vesicles, expression of CIC-5 in Xenopus oocytes elicited chloride currents. We now show that CIC-5 also gives rise to strongly outwardly rectifying anion currents when expressed in oocytes. They closely resemble CIC-5 currents with which they share a NO$_3^->Cl^->Br^->I^-$ conductance sequence that differs from that reported for the highly homologous CIC-3. Both CIC-4 and CIC-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 CIC-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 CIC-3 does not have similar effects on CIC-5. Moreover, a mutation at the end of D2 (S168T in CIC-5) changes ion selectivity, and a mutation at the end of D3 (E211A in CIC-5 and E224A in CIC-4) changes voltage dependence and ion selectivity. This shows that CIC-4 and CIC-5 can directly mediate plasma membrane currents.

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The CLC$^1$ family of chloride channels, originally defined by the CIC-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 CIC-1 cause myotonia (3, 4), mutations in CIC-Kb Bartter’s syndrome (5), and mutations in CIC-5 cause Dent’s disease (6).

Dent’s disease is an X chromosome-linked disorder and has two main symptoms: hypercalcicuria, 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, CIC-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 CIC-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, CIC-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 CIC-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 CIC-5 mutations found in Dent’s disease (6, 10–12), currents were either significantly decreased or abolished. In contrast to CIC-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 CIC-5. Mutations compromising CIC-5 function would then change those currents quantitatively without changing their characteristics. Given that CIC-5 plays a role in endocytosis (8), CIC-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, CIC-5 is the best understood member of the CIC-3/4/5 branch of the CLC$^1$ gene family. These three proteins are about 80% identical (9). In previous studies, we did not observe currents upon CIC-4 expression (14). While some groups reported that CIC-3 expression gave no currents (14, 15), others reported outwardly rectifying currents with an I$^-$ selectivity (16–19). The work of Duan et al. (18) suggests that CIC-3 represents the ubiquitous swelling-activated chloride channel. These authors introduced a mutation into CIC-3 that was equivalent to a mutation changing pore properties in other CLC channels (20–22) and found the expected changes in CIC-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, CIC-4 gave chloride currents when expressed in Xenopus oocytes or HEK293 cells. These resemble CIC-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 CIC-4 and CIC-5. Surprisingly, their properties differ significantly from those reported for CIC-3 (16–19), and we could not detect currents upon CIC-3 expression.
Experimental Procedures

ClC-4 and ClC-5 Constructs—A human ClC-4 cDNA was cloned into pTLN (23) or pcDNA (Promega, Madison, WI). It differed from the published sequence (24) (GenBankTM accession number X77197) at the following residues: Ala instead of Arg (GenBankTM accession number Z36844) and mouse (GenBankTM 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 DraIII 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 cDNA was transcribed from the constructs after linearization. 10–25 ng of cDNA 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 CaCl2, 0.33 mM NaHCO3, 0.82 mM MgSO4, 10 mM HEPES, pH 7.6). Two-electrode voltage-clamp measurements were performed at room temperature 2–5 days after injection using TurboTip 05 or 10 (Axon Instruments; Germany) and pClamp5.5 software (Axon Instruments). Currents were recorded in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, pH 7.4). For anion replacement, 80 mM Cl− was substituted by equivalent amounts of Br−, I−, NO3−, 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 pcDNA) using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer’s procedures. To identify transfectants, a green fluorescent protein construct (pEGFP; CLONTECH) was co-transfected. 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 MgCl2, 2 mM CaCl2, 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 mΩ tip diameter and filled with either high-CI intracellular solution (2 M KCl, 2 mM MgCl2, 2 mM EGTA, 5 mM HEPES, pH 7.4) or low-CI intracellular solution (120 mM NMDG-aspartate, 20 mM NMDG-Cl, 2 mM MgCl2, 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. 1a) 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 (contrasted to ClC-5, where currents begin to voltages more positive than +20 mV (9) and Fig. 1e)). ClC-4-induced currents are already visible at slightly more negative potentials and depend less steeply on voltage (Fig. 1e). Partial replacement of extracellular chloride by other anions indicated a NO3− > Cl− > Br− > I− conductance sequence (Fig. 1b). 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. 2a). 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. 2b). 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. 2c).

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 S250P 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. 3f). We mutated these critical residues to other amino acids (alanine and threonine). While S244A currents were similar to WT (Fig. 3a), 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 CIC-isoforms. Both mutations reduced the CI− > 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− ~ R relationship and an increase in the CI−/I− conductance ratio. However, this is not the case. While the N565K mutation slightly slowed the activation of ClC-5 currents (Fig. 3e), it did not change the overall rectification (Figs. 3e and 4a). It slightly increased the relative iodide conductance (Fig. 4a). 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 (GSGITE) at the end of D2 increases bromide and iodide conductances relative to chloride (27). The equivalent mutation (S168T) also reduces the iodine selectivity of ClC-5 (Fig. 4b). 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-
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 $\text{NO}_3^-$ (1.5) $\approx$ $\text{Br}^-$ (1.45) $>$ $\text{Cl}^-$ (1.0) = $\Gamma$ ratio for ClC-4(E224A), and a $\text{Br}^-$ (1.2) $>$ $\text{Cl}^-$ (1.0) = $\text{NO}_3^-$ $>$ $\Gamma$ (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
strongly rectifying channels with an \( \Gamma^+ > Cl^- \) selectivity (16–19). In previous experiments, we (9, 14) and others (15) were unable to obtain CIC-3 currents in \textit{Xenopus} oocytes. We have now repeated these experiments with human and guinea pig (18) CIC-3 cDNAs cloned into the expression vectors used in this study. We again failed to observe currents with human CIC-3 in \textit{Xenopus} oocytes. Attempts to express chloride currents with the guinea pig CIC-3 in transiently transfected HEK293 or NIH3T3 cells were unsuccessful as well.

**DISCUSSION**

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

We now demonstrate that both CIC-4 and CIC-5 directly mediate plasma membrane currents that are very similar. This includes their extreme outward rectification, the lack of significant tail currents, and the \( \text{NO}_2^- > Cl^- > Br^- > \Gamma^- \) conductance sequence at strongly depolarizing potentials. Both CIC-4 and CIC-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 \textit{Xenopus} oocytes (32). This is also unlikely in view of the very similar currents in different expression systems (\textit{Xenopus} oocytes and HEK293 cells).

The observed pH dependence of CIC-4 and CIC-5 possibly points to an important physiological role of these channels. CIC-5 is expressed in the endocytic 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 CIC 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 endocytic 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 CIC-5 fits well with a localization in the early endocytic pathway (8). An assignment for the physiological role of the pH dependence of CIC-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 CIC-4 or CIC-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 \textit{CLC} proteins can form heteromeric channels with novel properties (23), but we observed no qualitative changes when co-expressing CIC-5 with either CIC-4 or CIC-3 (not shown). CIC channels may also have as yet unknown \( \beta \)-type subunits.

The most compelling evidence for a direct channel function of CIC-4 and CIC-5 comes from our mutagenesis experiments. Several conservative amino acid exchanges slowed the activation of CIC-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 \( \beta \)-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 \textit{CLC} channels has not yet been defined. Point mutations at different positions change pore properties in CIC-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 CIC-5. A mutation at the end of D12 (CIC-5(N65K)) 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 (CIC-4(E2244A) and CIC-5(E211A)) had drastic effects on rectification of both channels and also slightly changed their conductance sequences. Several mutations in the same region in CIC-1 yielded channels with large inward currents (19), as did a mutation in the correspond-
FIG. 3. Mutants of CIC-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 CIC-5 and examined by whole-cell patch-clamp experiments. For comparison with WT currents, see Fig. 1d. Measurements were done as in Fig. 1d with low Cl\textsuperscript{−} intracellular solution, but the voltage range was between +120 mV and −100 mV. Time constants for current activation were determined to: WT, $\tau = 32 \pm 3$ ms; S244A, $31 \pm 2$ ms; S244T, $74 \pm 3$ ms; S520A, $68 \pm 2$ ms; S520T, 

A

S244A

B

S244T

C

S520A

D

S520T

E

N565K

F

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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 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 oocytes2 (26). Indeed, injection into *Xenopus* oocytes of cRNAs encoding several unrelated proteins

\[ \text{N. Wills, personal communication.} \]

\[ 78 \pm 4 \text{ ms; N565K, } 42 \pm 2 \text{ ms (values are means } \pm \text{ S.E.; } n = 5). \]

\[ a, \text{ topology model for CLC channels showing the position of amino acids mutated in this work. Note that ClC-4 has a longer amino terminus than ClC-5, leading to different numbering of equivalent positions. Terminology of domains D1 through D13 is based on Ref. 1, and topology is drawn according to the experimental results of Ref. 30. However, there are conflicting results (19) for the D4-D5 region.} \]

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**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. 1a. 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 NO3− (▲), 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.
can elicit similar outwardly rectifying chloride currents with an $\text{I}^- > \text{Cl}^-$ 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 $\text{I}^- > \text{Cl}^-$ 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 DI2 (N579K). The current-voltage relationship apparently changed from outwardly rectifying to linear and ion selectivity from $\text{I}^- > \text{Cl}^-$ to $\text{Cl}^- > \text{I}^-$ (18). This was expected since a mutation in ClC-5 (8), future studies should address the subcellular distribution of the highly homologous ClC-3 and ClC-4 channels. In view of the predominantly intracellular localization of ClC-3 (8), further 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. 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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