Pores Formed by Single Subunits in Mixed Dimers of Different CLC Chloride Channels*

Frank Weinreich and Thomas J. Jentsch‡
From the Zentrum für Molekulare Neurobiologie Hamburg, ZMNH, Hamburg University, Martinistrasse 85, D-20246 Hamburg, Germany

CLC chloride channels comprise a gene family with nine mammalian members. Probably all CLC channels form homodimers, and some CLC proteins may also associate to heterodimers. CIC-0 and CIC-1, the only CLC channels investigated at the single-channel level, display two conductances of equal size which are thought to result from two separate pores, formed individually by the two monomers. We generated concatemeric channels containing one subunit of CIC-0 together with one subunit of CIC-1 or CIC-2. They should display two different conductances if one monomer were sufficient to form one pore. Indeed, we found a 8-picosiemens (pS) conductance (corresponding to CIC-0) that was associated with either a 1.8-pS (CIC-1) or a 2.8-pS (CIC-2) conductance. These conductances retained their typical gating, but the slow gating of CIC-0 that affects both pores simultaneously was lost. CIC-2 and CIC-0 current components were modified by point mutations in the corresponding subunit. The CIC-2 single pore of the mixed dimer was compared with the pores in the CIC-2 homodimer and found to be unaltered. We conclude that each monomer individually forms a gated pore. CLC dimers in general must be imagined as having two pores, as shown previously for CIC-0.

The pore architecture of anion channels is still poorly known. Structure-function studies have been undertaken for a number of structurally unrelated chloride channel classes, such as cystic fibrosis transmembrane conductance regulator (1, 2), ligand-gated anion channels (3), and CLC channels (4). Sedimentation studies suggested that CIC-0 (5), CIC-1 (6), and a bacterial CLC protein (7) are dimers. For the bacterial CLC, this was confirmed by cross-linking experiments. In single-channel recordings, CIC-0 displays two conductance levels of equal magnitude. These gate independently, but are shut off together by a different, slow gating process. This led to the suggestion that CIC-0 is a “double-barreled” channel, which has two identical, largely independent pores (8). This model was confirmed by studies in which only of the subunits in the homodimer was mutated (9, 10). These channels displayed single-channel conductances that were compatible with one wild-type pore and one mutated pore. The important question if each subunit individually forms a pore has also been addressed for CIC-0. Concatemers with two mutant subunits suggested that one subunit forms one pore (10), although the presence of two mixed pores, formed by different parts of each subunit, could not be completely ruled out. However, the double-barreled structure of CLC channels has recently been questioned. The effect on whole cell-currents caused by the modification of cysteines in mutant CIC-1 channels led to the suggestion (11) that the two subunits of CIC-1 form a single pore that includes the D3-D4 region from each subunit. In single-channel records, however, CIC-1 displays a double-pore behavior comparable to that of CIC-0 (12). All CLC channels identified so far are homologous in the entire segment encompassing the 10–12 transmembrane domains. Hence, a common pore architecture must be assumed. This implies that either the “one-subunit/one-pore” model postulated for CIC-0 is valid for all members of this gene family, or it is valid for none of the channels, including CIC-0.

To demonstrate the functional and structural separation of individual pores in a CLC channel dimer, we constructed concatemers of two CLC channel monomers, linked in a head-to-tail fashion. The expression of concatenated subunits rather than coexpression of the corresponding monomers offers the advantage that only a single type of dimer will be formed. This approach has been used in the past to demonstrate separate pores in the CIC-0 channel (9, 10, 13). We now extend it to the study of concatemers composed of different CLC monomers, namely those of CIC-0, -1, and -2. Both CIC-1 and CIC-2 are highly homologous to CIC-0, with 54% and 49% identity at the amino acid level, respectively (14, 15). CIC-1 and CIC-2, which share 55% of sequence identity (15), have already been shown to form functional mixed dimers with altered properties in coexpression experiments (16).

Since CIC-0 has been studied extensively on the single-channel level (8, 13, 17, 18), its presence in the mixed channels may be demonstrated unambiguously by single-channel analysis. Only one study (12) showed single-channel recordings of CIC-1, and single CIC-2 channels have not yet been reported. In mixed concatemers of CIC-0, -1, and -2, we observed properties of both constituent pores in the macroscopic current. In single-channel recordings, we could clearly distinguish two different conductance levels that can be attributed to the pores of the constituent subunits. This demonstrates that one CLC subunit forms one pore, which retains most of its properties in a dimer irrespective of its partner. This is also the first time that single-channel traces of the CIC-2 pore are reported.

EXPERIMENTAL PROCEDURES

Construction of Concatemeric Channels and Expression in Xenopus Oocytes—To generate concatemeric channels, the stop codon of the N-terminal subunit was replaced with a PacI restriction site, which was then used to link it to the C-terminal subunit. The linker sequence consisted of four amino acids (L-I-K-A). Point mutations were intro-
Fig. 1. Macroscopic properties of mixed concatemers of CIC-0 and CIC-1. A, families of current traces, obtained with the voltage protocol depicted in the insert, are shown for the four possible combinations of concatemers consisting of CIC-0 and/or CIC-1. The CIC-1 concatemer clearly exhibits the properties of CIC-1, yet the two mixed concatemers are indistinguishable from the CIC-0 concatemer. B, the ratio of the tail current at +40 mV, obtained after a 7-s prepulse to the indicated potential, to the current obtained with a +40-mV prepulse is a measure of the hyperpolarization-activated slow common gate of CIC-0 (29). When this is compared for the different concatemers, the CIC-0 concatemer shows a considerable activation starting at ~−60 mV, whereas both mixed concatemers lack this activation by hyperpolarization. C, the sensitivity to the inhibitor 9-AC is shown for three concatemers consisting of CIC-1 and/or CIC-0(K519E) subunits, which have almost equal single-channel conductances. The CIC-0(K519E) concatemer has an apparent IC_{50} of 9.8 ± 0.1 mM, the CIC-1 concatemer of 8.2 ± 1.0 μM. In the mixed concatemer, a small fraction of the current (about 12%) is inhibitable by small concentrations of 9-AC (IC_{50} = 10.6 ± 11.7 μM) and the remainder is inhibited by much higher concentrations (IC_{50} = 10.7 ± 3.4 mM). Note that the 9-AC dependence of the CIC-1 current, in contrast to those of the homodimeric concatemers, is not well fitted by the function used to determine the IC_{50}. Data points in B and C represent the mean ± S.E. of three to six individual determinations. Error bars smaller than the symbol size are not shown.

The abbreviations used are: PCR, polymerase chain reaction; NMG, N-methyl-D-glucamine; 9-AC, 9-anthracene carboxylate; S, siemens.

RESULTS

Mixed Concatemers of CIC-1 and CIC-0—To test whether different CLC subunits may associate to form mixed pores with novel characteristics, or whether one pore is formed exclusively by one subunit, we constructed mixed concatemers of two CLC monomers. For simplicity, we will describe our results in the framework of the one-subunit/one-pore model and evaluate alternative models in the discussion section.

Using a four-amino acid linker sequence (see “Experimental Procedures”), CIC-0 and CIC-1 were linked in both possible orientations, i.e. CIC-1–CIC-1, CIC-0–CIC-1, and CIC-1–CIC-0. For comparison, homomeric concatemers of CIC-1 and of CIC-0 with the same linker sequence were constructed. All four concatemers could be expressed functionally in Xenopus oocytes (Fig. 1A). Apart from a reduced expression efficiency, no conspicuous differences were found between concatenated and monomeric CIC-0, in accordance with previous studies of concatenated CIC-0 (10, 13), which reported wild-type behavior for macroscopic and single-channel properties of the concatemer.
indicated that concatemerization per se did not alter channel properties.

The current amplitudes for the different concatemers differed significantly at the peak of expression (3 days after RNA injection). The slope conductance at 0 mV of the 0–0 concatemer (116 ± 23 μS) was about 4–5 times higher than for the 1–1 concatemer (26 ± 6 μS), and the two mixed concatemers had conductances of 67 ± 21 μS in the 1–0 and 159 ± 24 μS in the 0–1 orientation (n = 10–21). Protein levels were not measured, so the differences in steady-state current amplitudes may be caused either by a different conductance or by differences in expression level. In general, however, current amplitudes increased with the number of CIC-0 subunits in the concatemer, consistent with the higher single-channel conductance of CIC-0 (8 pS (Ref. 19)) as compared with CIC-1 (1.2 pS (Ref. 12)).

Both CIC-0 and CIC-1 are voltage-gated in more than one way. This has been described as a “fast” gate and a “slow” gate for CIC-0 (8, 18). Although the slow gate in CIC-1 is much faster than in CIC-0, the same terminology has been used for CIC-1 by Pusch and co-workers (12). The voltage dependence of the fast gate is qualitatively similar for CIC-0 and CIC-1. Both channels are closed by hyperpolarization, with the midpoint of the activation curve at about −100 mV for CIC-0 (20) and −20 mV for CIC-1 (21) in the oocyte system. Normal gating of CIC-1 is retained in the CIC-1–CIC-1 concatemer. However, no CIC-1-like currents were seen in the mixed concatemers with CIC-0. When a fast voltage protocol was used, their currents were very similar to CIC-0 (Fig. 1A). The steady-state current voltage dependence of the 1–0 and 0–1 concatemers was indistinguishable from that of the 0–0 concatemer (data not shown). Because of its lower single-channel conductance, the contribution of CIC-1 to the macroscopic current is expected to be small in mixed concatemers with CIC-0 (10–10%), but it should still be detectable. Although indistinguishable in their fast gating, both mixed concatemers differ from the CIC-0 concatemer by the absence of the hyperpolarization-activated slow gate, indicating that a different channel than in the 0–0 concatemer is formed (Fig. 1B).

The conclusion that the CIC-1 pore does not contribute significantly to macroscopic currents of the mixed concatemers is further supported by currents obtained from concatemers bearing the K519E mutation in the CIC-0 pore. Although the single-channel conductances of CIC-1 (1.2 pS (Ref. 12)) and CIC-0(K519E) (1 pS (Ref. 10)) are about equal, the macroscopic current of the mixed concatemers was very similar to the current obtained with the CIC-0(K519E) homodimer in terms of steady-state voltage dependence and open channel rectification. This was true irrespective of the order of the two subunits in the mixed concatemer (data not shown). However, the differential sensitivity of CIC-1 and CIC-0 to the inhibitor 9-AC (14, 22) may be exploited to demonstrate the presence of a CIC-1 conductance in the 1–0(K519E) concatemer. Extracellular 9-AC inhibited the CIC-1 concatemer with an apparent IC₅₀ of 8.2 ± 1.0 μM (Fig. 1C). This inhibition was not complete, because about 30% of the current remained at 500 μM 9-AC. The CIC-0(K519E) concatemer could only be inhibited by much higher 9-AC concentration, with an apparent IC₅₀ of 9.8 ± 0.8 mM. In the mixed concatemer, a biphasic inhibition was observed. A small fraction of the current (about 12%) was inhibited by similar concentrations of 9-AC as was the CIC-1 (IC₅₀ of 10.6 ± 11.7 μM), whereas the remaining current required equally high 9-AC concentrations as the CIC-0(K519E) to become blocked (IC₅₀ of 10.7 ± 3.4 ms). This indicates that the macroscopic current of the 1–0(K519E) concatemer is the sum of two current components with the same 9-AC sensitivity as the CIC-1 and CIC-0(K519E), respectively. Contrary to expec-

![Figure 2](image_url)

**Fig. 2. Single-channel properties of the CIC-1–CIC-0 mixed concatemer.** A, a continuous current trace of a single channel of the 1–0 concatemer, recorded at −100 mV, is shown. Four current levels (arrows) resulting from the presence of two different pores can be distinguished. The gating of both pores is independent of each other, because all possible gating transitions are observed between the four levels with equal likelihood, regardless of whether the other pore is open or closed (see "Results"). B, the single-pore conductance is determined from the current-voltage relationship in the range −80 to −160 mV. The conductance of the small pore (triangles) is 1.8 ± 0.1 pS; that of the large pore (squares) is 7.8 ± 0.2 pS, calculated from a linear fit to the data. Data points represent the mean ± S.E. of 3–12 individual determinations.
Separate Pores in Mixed CLC Channels

that lead to the bursting behavior normally associated with ClC-0 single-channel currents were not observed in the mixed concatemer.

Even in the absence of a clearly visible common gate, the gating of the pores might be interdependent. We therefore determined the open probability of the large pore in the 1–0 concatemer in relation to the open state of the small pore. The single-channel record was subdivided into sections of small pore open and closed events, and the open probability of the large pore calculated under both conditions. When the small pore was closed, the open probability (at −100 mV) of the large pore was 0.45 ± 0.03 (n = 5 patches). When the small pore was open, a value of 0.48 ± 0.02 was obtained, suggesting that the gating of the large pore did not depend on the open state of the small pore. The large pore open probability agrees with the corresponding value for individual pores in homomeric ClC-0, which is −0.45 at this voltage (10).

**Mixed Concatemers of ClC-2 and ClC-0**—The gating of ClC-2 differs significantly from ClC-1 and ClC-0. It opens very slowly upon hyperpolarization, is virtually closed at positive potentials, and can be opened by cell swelling and extracellular acidification (24, 25). An N-terminal inactivation domain (residues 21–39) was proposed to influence channel gating from the cytoplasmic side by a ball-and-chain mechanism (24). It is currently unclear how many of these inactivation domains are needed to gate a channel dimer. Possible movement restrictions of the second inactivation domain in the 2–2 concatemer apparently did not interfere with normal gating (compare Fig. 3A). However, when we generated mixed concatemers of ClC-2 and ClC-0, only the concatemer with the N-terminal ClC-2 moiety could be expressed functionally. In stark contrast to the ClC-1/ClC-0 concatemers, where the ClC-0 pore dominated the macroscopic current, current traces obtained with the ClC-2–ClC-0 mixed concatemer showed only a small depolarization-activated ClC-0 type conductance and a rather large hyperpolarization-activated ClC-2 type conductance (Fig. 3A).

To investigate whether the hyperpolarization-activated current was indeed carried by ClC-2 pores, we tested its modulation by external pH. The current increased by −40% upon lowering the pH by 1 unit, and decreased by the same amount upon raising the pH by 1 unit (25). ClC-0 is also weakly dependent on extracellular pH, but the moderate changes of ±1 pH unit employed here would not affect the steady-state current at the test potential of +40 mV (Ref. 17 and results not shown). This demonstrates that pH-dependent activation, which is a characteristic feature of ClC-2, is preserved in the 2–0 mixed concatemer.

Mutational analysis was used to identify the contribution of either pore to the macroscopic current. To this end, point mutations in either of the two subunits were inserted into the 2–0 concatemer and the resulting changes in the macroscopic current analyzed (Fig. 4). The ClC-2(K210Q) mutation accelerated the gating of homomeric ClC-2, resulting in a faster inactivation at depolarized potentials (data not shown). Inserting this mutation in the 2–0 concatemer (Fig. 4, left panel) accelerated the decay of the hyperpolarization-activated current upon switching to positive voltages. The K210Q mutation also reduced the hyperpolarization-activated current relative to the current at neutral potentials. This was determined from the ratio of the slope conductances at −120 mV and −40 mV, which was 2.43 and 1.33 for the traces shown in Figs. 3 and 4, respectively. A different effect was seen with the ClC-2(K566E) mutation, which caused outward rectification of the open-pore currents in the homomer (25). This effect is preserved in the...
mixed concatemer (Fig. 4, middle panel), since the current at negative voltages is significantly reduced in comparison with wild-type 2−0 currents. Finally, the ClC-0-like current could be suppressed by the introduction of the ClC-0(K519E) mutation, which greatly reduces the single-channel amplitude (10). The current of the 2−0(K519E) concatemer is only slightly different from that of the 2−2 concatemer (Fig. 4, right panel), indicating that a ClC-2 pore with wild-type gating behavior is present in the mixed concatemer.

Single-channel analysis of the 2−0 mixed concatemer is complicated by the fact that no single-channel currents of ClC-2 have been published. Macroscopic currents suggest that the ClC-2 pore should be open only at negative potentials, and noise analysis indicated a single-channel conductance of 2−3 pS (16). When single-channels of the 2−0 concatemer were recorded (Fig. 5), a pore conforming to these predictions was indeed found in association with a ClC-0 type pore. In all recordings that showed a single 2.8-pS conductance level, a smaller conductance of 2.8 pS was also found (7 patches). In contrast to the ClC-0 pore, which was always active and gated rapidly, the small pore opened only slowly after switching from positive to negative potential and closed quickly upon returning to positive potential. Once opened by negative voltage, very long open times, interrupted only by brief closings, could be observed (Fig. 5A, bottom trace).

To finally ascertain the identity of the small pore in the 2−0 concatemer, single-channel recordings of the 2−2 concatemer were performed. This revealed pores with a single-channel conductance of 26 ± 0.1 pS (Fig. 6) that gated similarly to the small pore in the 2−0 mixed concatemer. Again, positive voltage caused the pores to close, and upon switching to negative voltage, the pores re-opened only after a significant delay (compare Fig. 6B). Unlike the ClC-0, where the slow gating mechanism closes both pores simultaneously, ClC-2 showed no bursting behavior. This constant channel activity of ClC-2, in combination with its slow activation after a hyperpolarizing voltage step, did not allow us to unequivocally determine the minimum number of active pores, i.e. the pore stoichiometry of the homomeric channel.

DISCUSSION
Ever since the first double opening of the Torpedo electric organ voltage-gated chloride channel appeared on the chart recorder, the question whether this functional duplicity corresponded also to a structural duplicity, i.e. a two-pore channel, has been under debate. For the Torpedo channel, ClC-0, it has been answered in favor of two separate conduction pathways in terms of gating behavior (8, 17) and inhibitor blockade (26). The most stringent proof for this “double-barreled” model comes from the analysis of point mutations in mixed concatemers (9, 10, 13). On the other hand, the existence of a common gate that affects both pores simultaneously in ClC-0 (8, 27) and ClC-1 (12), together with dominant negative mutations in ClC-1 that alter channel gating (21) and dominant negative effects of biochemical modification of single monomers in mixed ClC-1 concatemers (11), suggest a functional interaction between both halves of the dimer. The cysteine modification studies of Fahlke and co-workers (11) were even taken as direct evidence for of a single conduction pathway in the dimeric channel, although neither true pore properties nor single-channel behavior were investigated.

The question we have asked is this: are pore properties such as single-channel conductance retained when subunits of different channels are expressed together in a single dimer? If they are, the double-pore arrangement observed in ClC-0 (8, 27) and ClC-1 (12), together with dominant negative mutations in ClC-1 that alter channel gating (21) and dominant negative effects of biochemical modification of single monomers in mixed ClC-1 concatemers (11), suggest a functional interaction between both halves of the dimer. The cysteine modification studies of Fahlke and co-workers (11) were even taken as direct evidence for of a single conduction pathway in the dimeric channel, although neither true pore properties nor single-channel behavior were investigated.

FIG. 4. Macroscopic properties of mutant ClC-2−ClC-0 concatemers. A, families of current traces obtained with the protocol shown in Fig. 3A for three different point mutations in the 2−0 concatemer. B, steady-state current-voltage relationships for the traces shown in A. The ClC-2(K210Q) mutation accelerates the decay of the hyperpolarization-activated current. The ClC-2(K566E) mutation renders the current outwardly rectifying. Finally, the ClC-0(K519E) mutation greatly reduces the depolarization-activated current, resulting in a conductance resembling that of the ClC-2 homodimer (compare Fig. 3).
of a single ClC-0 pore accompanied by a smaller pore. The small pore behaved like a ClC-1 pore in the 1–0 concatemer and like a ClC-2 pore in the 2–0 concatemer. Neither pore was encountered alone, but both pores were invariably found together. Moreover, the gating kinetics of the individual pores closely mimic the gating observed in homodimers of the respective subunit. This means that the structures responsible for pore formation and for voltage-dependent (fast) gating are present in any one subunit.

Other properties, however, are dependent on both pores in the dimer. This is clearly the case for the slow gate of the ClC-0, which is no longer seen in the macroscopic current if one ClC-0 subunit is replaced with a ClC-1 subunit. In the single-channel records of the mixed concatemers, two types of coordinated gating activity may be discerned, the direct transition between fully open and fully closed states, and the direct transition between small and large open levels. The former is nothing else but the slow gating well known in ClC-0, but the latter interlevel transitions would be missed in homomeric channels with two pores of equal conductance. Close inspection of the traces shown (Figs. 2A and 5A) seems to yield a few examples of either type of gating, i.e., coordinated opening/closing events as well as interlevel transitions. Considering the limited bandwidth of our recordings (2 kHz prior to filtering), these could result from incompletely resolved sequential gating events, but we cannot rule out the possibility that coordinated gating activity takes place in the mixed dimers with a low incidence. Since the frequency of these events is in any case too low to significantly alter channel behavior, we have not systematically investigated this example of subunit interdependence.

Furthermore, the contribution of the ClC-1 pore is very much reduced under voltage-clamp conditions when expressed alongside the ClC-0 pore, although both pores are clearly active in excised patches. This discrepancy may be due to the interaction with cytoplasmic cofactors or depend on the low [Cl] of the oocyte interior. Interestingly, in earlier studies of ClC-1 and ClC-2 coexpression (16), ClC-1 contributed little to the macroscopic current (and this most likely resulted from ClC-1 homodimers). Rather, the macroscopic current resembled that of a constitutively open ClC-2 channel. Quite different from the apparent suppression of ClC-1 in the 1–0 and 0–1 concatemers, ClC-2 dominates the macroscopic current in the 2–0 concatemer under whole cell conditions, indicating that ClC-0 is suppressed. Again, in the excised patch, ClC-0 and ClC-2 pores show normal gating.

Assuming that two separate pores are present in a channel consisting of two subunits, the question remains whether one pore is contained completely within a single subunit or formed by parts of each subunit. This question has been addressed in the past in concatemers of ClC-0 carrying two different mutations (10). Although these experiments were fully compatible with a one-subunit/one-pore arrangement, they could not entirely resolve the issue, because the pore structure of CLC channel is not known. The experiments with dimers of two different CLC channels that are reported here demonstrate that the basic channel properties of the monomer are not altered by its interaction with other subunits. This shows that a pore is formed entirely by a single CLC monomer.

We have interpreted our results based on the assumption that only two CLC subunits are required to form a functional channel, but is this justified? Our assumption is well supported by biochemical evidence, which suggests a dimeric structure for ClC-0 (5), ClC-1 (6), and a bacterial CLC homologue (7, 28). Nevertheless, a dimerization of the concatemers used in this
study (resulting in a dimer of dimers) cannot strictly be ruled out. If this should happen, dimers of the two constituent pores rather than mixed dimers could be formed, rendering the single-channel studies meaningless. The presence of single pores of each constituent channel type in the mixed dimers, however, argues against this possibility, for in a tetrameric arrangement, four pores should be present.

Can our results be explained in terms of a single-barreled channel, in which both subunits contribute to a single pore? This alternative model of CLC pore architecture was brought forward by Fahlike et al. (11) based on the interaction of single cysteine mutants of CIC-1 with mono- and bifunctional reagents. In the framework of this model, the two equal-sized conductance values observed in single-channel recordings must be regarded as subconductance states of a common pore. A pore consisting of two different CLC proteins, as is the case in our mixed concatamers, could then have two different subconductance states. However, it seems impossible that such subconductance states retain their conductance levels and gating properties they have in the respective homodimer, and that they gate independently of each other in the asymmetric heterodimer.

Taken together, our results argue for a common structural basis of all CLC channels, with a separate conduction pathway, i.e. a pore, in each subunit. The fundamental characteristics of channel activity, namely permeation of ions, mirrored in a defined single-channel conductance, and voltage-dependent gating transitions, are present in the monomeric channel and do not depend on the partner subunit. Any CLC dimer, therefore, must be viewed as an association of two basically independent pores. This does not exclude the possibility that some CLC channels are monomers. However, since the dimeric structure found in the bacterial channel (7) appears to be conserved in the mammalian channels, this seems unlikely. Last but not least, we have shown on the single-channel level that CIC-2 is a slowly gating, hyperpolarization-activated channel of 2–3 pS single-channel conductance, in agreement with prior studies of macroscopic currents. This enables a comparison with single-channel recordings from native tissues in which CIC-2 is expressed.

What are the consequences of the one-subunit/one-pore arrangement? One important feature of such a pore architecture is the mechanism by which mutations may affect channel function. In potassium channels, where four subunits contribute equally to a single pore, mutations in the pore as well as in other parts of the protein often have dominant negative effects. On the other hand, mutations in CLC channels will only show a dominant phenotype if they affect a common gating mechanism or if they lead to a retention or misprocessing of heteromorphic channels before they reach their target membrane. This observation is consistent with the analysis of a dominant negative mutation in CIC-1 causing myotonia congenita (12), which was found to affect the common (slow) gating but not the individual (fast) gating of the channels. Elucidating channel structure may therefore be an important tool for the understanding of mechanisms of pathogenesis in human inherited diseases. The double pore arrangement further implies that the design of dominant-negative mutants, which could be useful in cell biological or transgenic approaches, will not be an easy task for all members of the CLC family.

Acknowledgments—We thank Michael Pusch for valuable advice, Siegfried Waldegger for critical reading of the manuscript, and Sven-Eric Jordt for the construction of some of the mutants used in this study.

REFERENCES
1. Akabas, M. H., Cheung, M., and Guinamard, R. (1997) J. Bioenerg. Biomembr. 29, 453–463
2. Sheppard, D. N., and Welsh, M. J. (1999) Physiol. Rev. 79, S23–S45
3. Bormann, J., Rundstrom, N., Betz, H., and Langosch, D. (1993) EMBO J. 12, 3729–3737
4. Jentsch, T. J., Friedrich, T., Schriever, A., and Yamada, H. (1999) Pflugers Arch. 437, 783–795
5. Middleton, R. E., Pheasant, D. J., and Miller, C. (1994) Biochemistry 33, 13189–13196
6. Fahlike, C., Knittle, T., Garnett, C. A., Campbell, K. P., and George, A. L. Jr. (1997) J. Gen. Physiol. 109, 93–104
7. Maduce, M., Pheasant, D. J., and Miller, C. (1999) J. Gen. Physiol. 114, 713–722
8. Miller, C. (1982) Philos. Trans. R. Soc. Lond. B Biol. Sci. 299, 401–411
9. Middleton, R. E., Pheasant, D. J., and Miller, C. (1998) Nature 393, 337–340
10. Ludewig, U., Pusch, M., and Jentsch, T. J. (1996) Nature 384, 340–343
11. Fahlike, C., Rhodes, T. H., Dessai, R. R., and George, A. L., Jr. (1998) Nature 394, 687–690
12. Saviane, C., Conti, F., and Pusch, M. (1999) J. Gen. Physiol. 113, 457–468
13. Ludewig, U., Pusch, M., and Jentsch, T. J. (1997) Biophys. J. 73, 789–797
14. Steinmeyer, K., Thiemann, A., and Jentsch, T. J. (1991) Nature 354, 301–304
15. Thiemann, A., Grunder, S., Pusch, M., and Jentsch, T. J. (1992) Nature 356, 57–60
16. Lorenz, C., Pusch, M., and Jentsch, T. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13362–13366
17. Hanke, W., and Miller, C. (1983) J. Gen. Physiol. 82, 25–45
18. Bauer, C. K., Steinmeyer, K., Schwarz, J. R., and Jentsch, T. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11052–11056
19. Ludewig, U., Jentsch, T. J., and Pusch, M. (1997) J. Gen. Physiol. 110, 165–171
20. Pusch, M., Ludewig, U., Rehfeldt, A., and Jentsch, T. J. (1995) Nature 373, 527–531
21. Pusch, M., Steinmeyer, K., Koch, M. C., and Jentsch, T. J. (1995) Neuron 15, 1455–1463
22. Astill, D. S., Ryehkov, G., Clarke, J. D., Hughes, B. P., Roberts, M. L., and Bretag, A. H. (1996) Biochim. Biophys. Acta 1290, 178–186
23. Ryehkov, G. Y., Pusch, M., Astill, D. S., Roberts, M. L., and Bretag, A. H. (1996) J. Physiol. 497, 423–435
24. Grunder, S., Thiemann, A., and Jentsch, T. J. (1992) Nature 360, 759–762
25. Jentsch, T. J., and Miller, C. (1992) J. Gen. Physiol. 109, 165–116
26. Miller, C., and White, M. M. (1984) J. Gen. Physiol. 789–797
27. Lin, Y. W., Lin, C. W., and Chen, T. Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7722–7725
28. Lin, Y. W., Lin, C. W., and Chen, T. Y. (1999) J. Gen. Physiol. 114, 1–12
29. Purdy, M. D., and Wiener, M. C. (2000) FEBS Lett. 466, 26–28
30. Pusch, M., Ludewig, U., and Jentsch, T. J. (1997) J. Gen. Physiol. 109, 105–116