Residues Lining the Inner Pore Vestibule of Human Muscle Chloride Channels*

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Christoph Fahlke‡§, Reshma R. Desai‡, Niloufar Gillhani, and Alfred L. George, Jr.‡

From the ‡Department of Pharmacology and Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, SRWTH Aachen, Institute of Physiology, 52057 Aachen, Germany, and §Centro de Estudios Científicos (CECS), Avenida Prat 514, Valdivia, Chile

Chloride channels belonging to the CIC family are ubiquitous and participate in a wide variety of physiological and pathophysiological processes. To define sequence segments in CIC channels that contribute to the formation of their ion conduction pathway, we employed a combination of site-directed mutagenesis, heterologous expression, patch clamp recordings, and chemical modification of the human muscle CIC isoform, hClC-1. We demonstrate that a highly conserved 8-amino acid motif (P3) located in the linker between transmembrane domains D2 and D3 contributes to the formation of a wide pore vestibule facing the cell interior. Similar to a previously defined pore region (P1 region), this segment functionally interacts with the corresponding segment of the contralateral subunit. The use of cysteine-specific reagents of different size revealed marked differences in the diameter of pore-forming regions implying that CIC channels exhibit a pore architecture quite similar to that of certain cation channels, in which a narrow constriction containing major structural determinants of ion selectivity is neighbored by wide vestibules on both sides of the membrane.

The CIC family of voltage-gated Cl^- channels represents the largest known gene family coding for anion channels (1–3). At least nine human isoforms (CIC-1 to CIC-7, CIC-Ka, and CIC-Kb) are expressed in various tissues and play important roles in the function of various organs. Mutations in the genes coding for three CIC isoforms cause inherited human diseases. CLCN1 represents the genetic locus for myotonia congenita (4, 5), a muscle disease characterized by stiffness upon sudden forceful movement. Mutations in CLCN5 cause Dent’s disease, an inherited renal disorder associated with hypercalciuria, nephrolithiasis, and low molecular weight proteinuria (6). Genetic alterations of CLCNKB are responsible for type III Bartter’s syndrome, a salt-wasting renal tubular disorder causing hypovolemia, hypotension, and hypotension (7).

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† Recipient of a Heisenberg-Stipendium from the German Research Foundation (Grant Fa 301/3-1) for part of this work. To whom correspondence should be addressed: Institut fur Physiologie, RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany. Tel: 49 241 80 888 10; Fax: 49 241 8888 434; E-mail: cfahlke@physiology.rwth-aachen.de.

1 The abbreviations used are: hCIC-1, human CIC-1; MTSET, methanethiosulfonate; MTSES, methanethiosulfonate ethylmethylammonium; WT, wild type.
experimental tests to identify pore-forming regions of CIC channels. We have previously employed a combination of several methods to identify two regions within hCIC-1 that line a portion of the ion conduction pathway and represent major structural determinants of ion selectivity. A highly conserved 8-amino acid sequence present in every known eukaryotic CIC channel (GKXGXPXXH) lines the most narrow part of the CIC pore. The P1 region is a major determinant of the binding affinities for anions (15) and is responsible for the high anion to cation selectivity of hCIC-1 (18). Within the fifth transmembrane domain (D5), we have identified another highly conserved amino acid region, GVLFSI in hCIC-1 (designated as P2), that together with P1 lines the narrow part of the pore (18). In the present study, we extend our investigation to another highly conserved segment, the linker region between transmembrane domain 2 (D2) and 3 (D3). This segment was previously demonstrated to have a single residue that can influence single channel conductance of CIC-0 (16), but a systematic evaluation of this region was lacking. We now provide further evidence that this segment is important for ion selectivity, and we provide evidence that this region contributes to the formation of a wide inner pore vestibule.

MATERIALS AND METHODS

Mutagenesis and Construction of Heterodimers—Site-specific mutants were constructed using recombinant polymerase chain mutagenesis. Monomeric constructs were assembled in the expression construct pRoCMV-hCIC-1, and dimers were constructed as described previously (19). We then sequenced in both constructs regions modified by polymerase chain reaction completely to exclude polymerase errors. At least two independent recombinants were examined functionally for each mutant or dimer. Transient transfection of tsA201 was performed as described previously (15, 19).

Electrophysiology—Standard whole-cell, inside-out, or outside-out patch clamp recordings (20) were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Pipettes were pulled from borosilicate glass and had resistances of 0.8–2.2 MΩ. More than 80% of the series resistance was compensated by the equivalent circuit model. The calculated voltage error due to series resistance was always <5 mV. Currents were filtered with an internal 4-pole Bessel filter with a 1-kHz filter roll-off (−3 dB) and digitized with sampling rates of 5 kHz using a Digidata AD/DA converter (Axon Instruments, Foster City, CA). Cells were clamped to 0 mV for at least 5 s between two test sweeps. The components of the solutions were as follows: extracellular solution (in mM), NaCl (140), KCl (4), CaCl 2 (2), MgCl 2 (1), HEPES (5), pH 7.4; intracellular (in mM), NaCl (130), MgCl 2 (2), EGTA (5), HEPES (10), pH 7.4. Anion permeability ratios were determined as described previously (15). Data were analyzed by a combination of pClamp (Axon Instruments, Foster City, CA) and SigmaPlot (Jandel Scientific, San Rafael, CA) programs. All data are shown as means ± S.E. from at least three different experiments.

Modification with MTS Reagents—2-Aminoethyl-methanethiosulfonate (MTSET) and ethylsulfonate (MTSES) were obtained from Toronto Research Chemicals (New York, Ontario, Canada). Stock solutions (0.1 M) were prepared in distilled water, stored at −20 °C, and diluted into the bath solution immediately before use. Cells/patches were held at 0 mV and stimulated every 5 s to voltage steps to +75 mV followed by −105 mV. Typically, 20 cycles were employed to assess control values (Imodified). Then MTS-reagents were applied to cells/patches by moving the cell/patch into the stream of a silane-treated macropipette filled with MTS-containing solution. After 3 min or after reaching a steady-state current amplitude, cells/patches were moved out of the stream to visualize reversible effects and to measure the current amplitude after irreversible modification (Imodified). The relative current reduction was measured as 1 − Imodified/Icontrol. The time course of modification was fit with a single exponential giving the time constant of modification. The pseudo-first order rate constant was calculated as the inverse of the modification time constant. Dividing by the concentration of the MTS reagents provided the second order rate constants. The charge selectivity of the access pathway for MTS reagents was calculated by dividing the second order reaction rates for MTSES by the rate for MTSET and normalizing this value to the relative reactivity of the two reagents with thiols in aqueous solution 0.08 (21). For experiments testing the effect of SCN− on MTS modification, 50 mM NaCl were substituted by equimolar amounts of NaSCN.

RESULTS

D2-D3 Linker Lines the Inner Mouth of the hCIC-1 Pore—All known CIC channels exhibit a conserved sequence motif between transmembrane domains D2 and D3 that has the sequence GSGIPEMK in hCIC-1 (Fig. 1). Earlier experiments demonstrated that an amino acid exchange within this region (S123T) changes the single channel amplitude and the conductance sequence of CIC-0 (16). The high degree of sequence conservation together with the observed effect of a single point mutation on CIC-0 conductance properties makes this segment (P3) a candidate pore-forming region. To investigate further the role of P3 in formation of the ion conduction pathway, we constructed single cysteine substitutions for each residue in the region and evaluated these mutants using the patch clamp technique and chemical modification with cysteine-specific methanethiosulfonate (MTS) reagents.

Fig. 2 illustrates representative macroscopic current recordings from each of the 8 single cysteine mutants, obtained using either whole-cell (Fig. 2A) or excised inside-out recordings from transiently transfected tsA201 cells (Fig. 2, B–H). All mutants were functional in transiently transfected tsA201 cells exhibiting large whole-cell current levels with maximum outward current amplitudes of several nA. With the exception of G188C, all mutants expressed at levels high enough to permit recording of large amplitude macroscopic currents in excised patches. Except for slight alterations in the voltage dependence of activation and the time course of deactivation (data not shown), all displayed gating properties very similar to wild-type (WT)
hClC-1 (22). In addition, all mutants exhibited an inwardly rectifying instantaneous current-voltage relationship that is characteristic of hClC-1. We conclude that cysteine substitutions within P3 do not disturb the general functional behavior of hClC-1, and this provides evidence that the mutations do not cause major structural rearrangements in the channel.

To test for alterations of the relative anion permeability sequence, reversal potentials were determined from cells internally perfused with standard internal solution and bathed in sequence, reversal potentials were determined from cells inter-
and that the access pathway to thiol side chains within this strate that parts of P3 are in contact with the aqueous phase yields the relative anion to cation selectivity of the access two reagents in aqueous solution (21), and this calculation of the partial substitution of Cl\(^-\) effectively block chloride currents. We studied the effect of within the pore by anions. Several anions bind more tightly with substituted cysteines will be impaired under experimental conditions that favor occupation of the particular binding site and that Gly-190 is located near an anion-binding site, but obviously anions do not bind as so close to this side chain but the reaction with E193C unchanged. We conclude that P3 contributes to an anion-binding site. If MTSET react with a residue located elsewhere within the pore, electrostatic interactions of its positive charge with the bound anion are expected to boost the access of MTS reagents and will produce opposite results.

For these experiments, we studied Cys-190 and Cys-193. Fig. 4 shows results for these two P3 cysteines as well as Cys-231 in P1. Several lines of evidence indicate that Lys-231 in P1 makes direct contact with permeating anions (18), and Cys-231 was therefore used as a control. Partial substitution of chloride by SCN\(^-\) completely abolishes the effects of Cys-231 modification suggesting that SCN\(^-\) protects this thiol group. For the thiol side chains substituted within P3, the effect of SCN\(^-\) was clearly distinct. Cys-190 as well as Cys-193 were modified in the presence of SCN\(^-\). For Cys-190, the extent of current reduction and the reaction rate during MTS treatment was significantly (\(p < 0.01\)) decreased. In contrast, for E193C, neither the reaction rates nor the degree of current reduction was altered in the presence of SCN\(^-\).

SCN\(^-\) as a weak nucleophile could react with MTS reagents, reduce their concentration, and thus cause a decreased apparent reaction rate. Although we cannot exclude that any of the applied MTS reagents reacted with SCN\(^-\), the marked difference of the SCN\(^-\) effect on the MTS reaction with different substituted cysteines demonstrates that this is not the major action of this anion. SCN\(^-\) completely abolishes the reaction of MTS with K231C; it reduces the reaction with G190C and leaves the reaction with E193C unchanged. We conclude that Lys-231 directly contributes to the formation of an anion-binding site and that Gly-190 is located near an anion-binding site, but obviously anions do not bind as so close to this side chain that the MTSET compounds cannot reach the thiol group. Our results do not support the notion that Glu-193 plays a role in anion binding within the CIC pore.

### Functional Interaction of P3 Regions—By using chemical

Table I

| P/P3 | P/SCN/P3 | P/NO3/P3 | P/B/P3 | Selectivity sequence |
|------|----------|----------|--------|----------------------|
| WT   | 0.34 ± 0.01 | 0.92 ± 0.04 | 0.53 ± 0.03 | 0.59 ± 0.06 | Cl > SCN > Br > NO3 > I |
| G188C | 2.2 ± 0.3 | 3.3 ± 0.6 | 0.55 ± 0.1 | 0.57 ± 0.06 | SCN > I > Cl > Br > NO3 |
| S189C | 2.0 ± 0.1 | 6.1 ± 0.8 | 0.5 ± 0.1 | 0.65 ± 0.03 | SCN > I > Cl > Br > NO3 |
| G190C | 0.23 ± 0.01 | 1.7 ± 0.1 | 0.36 ± 0.01 | 0.6 ± 0.03 | SCN > Cl > Br > NO3 > I |
| I191C | 0.33 ± 0.04 | 0.95 ± 0.06 | 0.34 ± 0.04 | 0.53 ± 0.01 | Cl > SCN > Br > NO3 > I |
| P192C | 0.28 ± 0.06 | 1.4 ± 0.06 | 0.24 ± 0.04 | 0.5 ± 0.01 | SCN > Cl > Br > I > NO3 |
| E193C | 0.16 ± 0.02 | 1.8 ± 0.1 | 0.35 ± 0.04 | 0.45 ± 0.01 | SCN > Cl > Br > NO3 > I |
| M194C | 0.21 ± 0.04 | 1.3 ± 0.03 | 0.5 ± 0.07 | 0.49 ± 0.04 | SCN > Cl > NO3 > Br > I |
| K195C | 0.26 ± 0.02 | 1.2 ± 0.1 | 0.22 ± 0.06 | 0.48 ± 0.03 | SCN > Cl > Br > NO3 |

Fig. 3. **Substituted cysteine accessibility experiments.** A, relative current change for substituted cysteine between S189C and K195C following application of intracellular MTSES (open bars) or MTSET (solid bars). For G188C, G190C, and I191C the relative changes of the instantaneous current amplitude at −105 mV are shown; for the remaining mutants the relative current amplitude changes at the +75 mV step. B, reaction rates for modification of these cysteines by MTSES or MTSET. C, relative anion selectivity of the access pathway for MTS reagents to four different substituted cysteines. All data were obtained from excised inside-out (for application of MTS reagents to the intracellular membrane side) or outside-out (for application of MTS reagents to the extracellular membrane side) patch clamp recordings.

We obtained second-order rate constants for modification with MTSES and MTSET (Fig. 3B). Rate constants for all reactions are well below the values observed for the modification of thiols in aqueous solution (21), and this observation suggests that the apparent access to the aqueous solution is the rate-limiting step. The apparent reaction rates can thus be used to judge whether the reagent diffuses through an anion-selective or unselective entry pathway. Because MTSES (anionic) and MTSET (cationic) have similar molecular diameters, differences in reaction rates of a given cysteine mutant with these two reagents are most likely caused by an electrostatic interaction, with regions of the channel protein determining the selectivity of the pore entrance as well as with the negatively charged thiolate form of the substituted cysteine with which the MTS compound reacts (21). To obtain the anion to cation selectivity of the access pathway, one has to correct for the latter component. For this reason, the measured rate constant ratio (\(k_{\text{MTSES}}/k_{\text{MTSET}}\)) is normalized to the relative reactivity of the two reagents in aqueous solution (21), and this calculation yields the relative anion to cation selectivity of the access pathway. For the tested substituted thiols within P3, we obtain values between 9.4 and 120 (Fig. 3C). These results demonstrate that parts of P3 are in contact with the aqueous phase and that the access pathway to thiol side chains within this region is anion-selective.

To test whether P3 contributes to the formation of an anion-binding site, we performed chemical modification experiments in the absence and presence of thiocyanate ions (SCN\(^-\)). If accessible side chains within P3 are in contact with permeating anions, one would expect that the reaction of MTS reagents with substituted cysteines will be impaired under experimental conditions that favor occupation of the particular binding site within the pore by anions. Several anions bind more tightly than chloride to sites within the CIC-1 ionic pore (8, 10) and effectively block chloride currents. We studied the effect of partial substitution of Cl\(^-\) with SCN\(^-\) on the reaction of substituted cysteines within P3 using the positively charged MTSET. If SCN\(^-\) binds close to the substituted cysteine, MTSET might be unable to react if the thiol side chain is shielded by bound SCN\(^-\) nearby. A decrease of either the degree of current reduction or the second-order reaction rate constants would indicate that P3 contributes to an anion-binding site. If MTSET reacts with a residue located elsewhere within the pore, electrostatic interactions of its positive charge with the bound anion are expected to boost the access of MTS reagents and will produce opposite results.
modification of homo- and heterodimeric cysteine-substituted channels, we have recently provided evidence that accessible side chains within P1 of both subunits of a single channel interact with each other in a way that would be expected if they jointly form a single vestibule (24). To test whether such an interaction also takes place between the two P3 regions, we employed a similar experimental strategy.

We engineered tandem constructs (19) to express a homogenous population of heterodimeric single cysteine-substituted channels. Fig. 5, A—C, illustrates representative current recordings from heterodimeric WT-G190C channels under standard conditions (Fig. 5A), during modification by MTSES (Fig. 5B) and afterward (Fig. 5C). After reaching steady-state conditions following MTSES modification, a substantial current component carried by WT-G190C remains (Fig. 5C). By contrast, modification of the double cysteine-substituted channel causes an almost complete disappearance of the typical hClC-1 gating phenotype (Fig. 5D). This outcome is different from that of experiments with cysteine-substituted channels within the P1 region (24) where chemical modification of homo- and heterodimeric channel causes identical functional changes. At first glance, these results appear to support the idea that each P3 region of a functional hClC-1 dimeric channel forms an independent ion conduction pathway. However, two lines of evidence demonstrate that this is not the case. A comparison of the time course of modification (Fig. 5E) demonstrates that the reaction is significantly slower in the heterodimeric channel than in the homodimeric double cysteine-substituted channel. This result is in clear contrast to the predictions of a double-barreled channel for which the rate constants of modification of a certain cysteine should be unaffected by substitutions in the other protochannel. Whereas for MTSES modification there is a significant component of unmodified current, the unblocked current levels after MTSET modification are indistinguishable for homo- and heterodimeric channel (Fig. 5E). This result is consistent with previous observations (19), which showed that the rate of modification of a single cysteine in a double cysteine-substituted channel is not significantly different from the rate of modification of a single cysteine in a single cysteine-substituted channel.

Fig. 6 illustrates the modification of P192C and E193C heterodimeric channels. In this case, the modification of the double cysteine-substituted channel causes a significant decrease in current amplitude (Fig. 6A), whereas the modification of the single cysteine-substituted channel causes a smaller decrease in current amplitude (Fig. 6B). This result is consistent with the idea that each P3 region of a functional hClC-1 dimeric channel forms an independent ion conduction pathway. However, two lines of evidence demonstrate that this is not the case. A comparison of the time course of modification (Fig. 6C) demonstrates that the reaction is significantly slower in the heterodimeric channel than in the homodimeric double cysteine-substituted channel. This result is in clear contrast to the predictions of a double-barreled channel for which the rate constants of modification of a certain cysteine should be unaffected by substitutions in the other protochannel. Whereas for MTSES modification there is a significant component of unmodified current, the unblocked current levels after MTSET modification are indistinguishable for homo- and heterodimeric channel (Fig. 6D). This result is consistent with previous observations (19), which showed that the rate of modification of a single cysteine in a double cysteine-substituted channel is not significantly different from the rate of modification of a single cysteine in a single cysteine-substituted channel.
erodimeric and homodimeric channels by MTS reagents. In all cases there are different effects on macroscopic currents through heterodimeric versus homodimeric channels. MTS modification of heterodimeric channels is not a simple superposition of the modification of cysteine-tagged pores with a current component by WT pores that are not modified. Similar to the other tested cysteines in the P1 and P3 regions, cysteine modification in one subunit does not cause functional effects that are independent from the other subunit.

The results obtained for single and double cysteine modification in P3 support the idea that this region in both subunits somehow interacts with the contralateral corresponding region, similar to that observed for the P1 region. Nevertheless, the functional effects of Cys-190 modification with MTSES in a heterodimeric channel are remarkably different from similar results obtained within the P1 region. A possible interpretation of these differences would be that the portion of the conduction pathway formed by P3 is wide enough to accommodate a covalently linked MTSES molecule and still permit ion flux. We tested this possibility by using different cysteine-specific reagents with larger substituents.

**Dimensions of the hClC-1 Pore**—Fig. 7 shows the results of chemical modification of four cysteine side chains protruding into the pore of CIC channels by the cysteine-specific reagents, qBr and MTS-PTrEA. MTS-PTrEA is a cubic molecule with a length of 8 Å, and qBr exhibits a dimension of more than 6 × 10 × 12 Å (25). Two cysteines are accessible to both reagents (K231C, G190C), E193C is only accessible to MTS-PTrEA, and H237C cannot be reached by either compound. As all these side chains are accessible to smaller cysteine-reactive reagents, the most likely possibility is that these large agents cannot bind because of steric hindrances. The dimensions of these agents therefore provide estimates for the proportions of the ionic pore of hCIC-1.

**DISCUSSION**

**P3 Is a Pore-lining Segment**—By using a combination of site-directed mutagenesis, heterologous expression, and chemical modification, we have demonstrated the following: 1) mutations within the highly conserved P3 region between transmembrane domains D2 and D3 affect anion selectivity of hCIC-1; 2) cysteine substitution experiments indicate that this segment is accessible to internally applied hydrophilic MTS reagents; 3) the internal access pathway of MTS reagents to P3 is anion-selective; and 4) SCN⁻ impairs MTS modification of a P3 thiol side chain (Cys-190). These results are all consistent with the idea that P3 forms part of the ionic conduction pathway of the hCIC-1 pore.

There are qualitative differences of the results obtained with P3 residues and those with P1 or P2 residues (18). For substituted cysteines within P1, the modification of a single cysteine decreases the current amplitude to zero levels, whereas in WT-G190C heterodimeric channels, modified by MTSES, a substantial amount of residual current could be observed. Moreover, whereas occupation of anionic binding sites prevents chemical modification of thiol side chains within P1, for residues belonging to the P3 region either no effect (E193C) or a less pronounced effect (G190C) could be observed. These results suggest that P3 may not contribute to the formation of the narrow part of the hCIC-1 pore and most probably is not part of an anionic binding site. A likely explanation for these results may be that P3 forms part of a wide internal vestibule.

**Estimating the Dimensions of the CIC Pore**—Studying interactions with high affinity pore blockers has provided many insights into the structure of the ion conduction pathway of many ion channels, most notably for organic compounds (26–28) or peptide blockers (29–31) with voltage-gated potassium channels. There are currently no specific blockers for CIC channels precluding employment of this approach. The use of cysteine-specific reagents has provided an alternative strategy for gaining insights into the dimensions of the ion conduction pathway of CIC channels.

Our earlier experiments with internal and external MTSES and MTSET demonstrated that there is a major constriction between Lys-231 and His-237, most probably the most narrow part of the CIC channel pore, and that this constriction is less than 6 Å (18). In agreement with these findings, experiments with various permeant anions provided evidence that this narrowing has a diameter between 4 and 6 Å (8, 10). The use of MTS-PTrEA and qBr now reveals that the pore diameter is wider than 10 Å at the level of Lys-231 and G190C and that Glu-193 and His-237 are located in pore regions that are more

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**Figure 6.** Distinct MTS modification of homo- and heterodimeric channels. A, P192C; B, WT-P192C; C, E193C; and D, WT-E193C. Inside-out patch clamp recordings and patches were held at 0 mV and stimulated every 5 s to voltage steps to +75 mV followed by −105 mV. Current responses for different times after MTS applications are superimposed. For A and B, 2 mM MTSET were applied, and for C and D, 50 μM MTSES.

**Figure 7.** Modification of cysteine-substituted hClC-1 channels with MTS-PTrEA and qBr. A, relative current reduction; and B, second order rate constants.
restricted. From our data, it would appear that CIC channels exhibit the same hourglass pore architecture as other classes of ion channels with a constriction displaying the major selectivity neighbored on both sides with wide vestibules. This architecture effectively reduces the passage of the ion through a low dielectric constant medium and thus significantly diminishes the resistance of the ion conduction pathway (32).

The result with Cys-231 is surprising given that two Cys-231 residues in a homodimeric CIC channel are close enough to form disulfide bridges (24). To account for these disparate observations, we suggest that the pore wall of CIC channels around position 231 is very flexible. Flexibility of ionic pores has been proposed to account for a stepwise dehydration of ions while entering the narrow part of an ion channel or carrier (33, 34), and experiments with oxidizing agents have recently directly demonstrated such an elasticity for the outer mouth of sodium channels (35).

For hCIC-1, this interpretation gives a structural basis for two other previous experimental results. An investigation of the blocking action of iodide suggested that there are conformational changes of the ionic pore of hClC-1 (8). A flexible pore region around Lys-231 could account for this finding because of the functional importance of this residue (18). Moreover, SCN− exerts a puzzling effect on ion conduction through hCIC-1. External SCN− blocks chloride currents in the negative and low positive voltage range, but at very positive potentials there is an outwardly rectifying current carried by SCN ions (10). Such a behavior (usually denoted as “punch through” effect) can be explained with a flexible pore (36, 37).

Pore Stoichiometry of CIC Channels—In 1982, Miller (38) suggested a novel and unique pore architecture for the CIC-0 chloride channel. Based on single channel recordings, he proposed that these channels exhibit two identical and functionally independent protopores (“double-barreled shotgun” model). In the following years, Miller and colleagues provided several lines of evidence supporting this concept (39, 40). Later experiments with heterodimeric CIC-0 channels in which the primary sequence of one subunit was mutated at two specific residues (16, 41) demonstrated that the unitary conductance as well as the ion selectivity of each subconductance state is determined by only one subunit supporting Miller’s original proposal. The results of the work contained in the present paper and those of an earlier study (24) now reveal that two regions that are critical for the ion conduction in hCIC-1 exhibit a behavior that reveals a functional interaction between the two subunits. These experiments are inconsistent with the proposed functional independence of the two protopores and appeared to be more consistent with a single conduction pathway than with the original double-barreled shotgun hypothesis. The different outcome of experiments performed with CIC-0 and hCIC-1 cannot be explained by isoform-specific differences in pore stoichiometry. As CIC-0 and hCIC-1 share high sequence identity, corresponding mutations in both isoforms cause similar functional alterations, and both isoforms display equally spaced subconductance states (38, 42); there is little doubt that CIC-0 and CIC-1 exhibit an identical quaternary structure of the pore.

These two experimental results represent a novel and interesting feature of CIC-type chloride channels. They either exhibit a single pore in which subconductance states occur that are completely independent, and we have at present only suggestions how this can happen (9), or they exhibit two ion conduction pathways that are independent in certain aspects but clearly interact in others. Another possibility is a combination of both, the existence of one or two common vestibules connected by two distinct conduction pathways. A definitive answer to this open question will provide important insights into the function of CIC-type chloride channels.

Conclusion—We have identified a segment (P3) that contributes to the formation of a wide internal vestibule of CIC channels. Experiments with cysteine-specific reagents of different size revealed that CIC channels exhibit a pore architecture quite similar to cation channels, with a narrow region that is important for anion-cation selectivity in the center and wide vestibules on both sides. These experiments provide novel insights into the architecture and the function of the pore of CIC-type chloride channels.

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