Mechanism of Inverted Activation of CIC-1 Channels Caused by a Novel Myotonia Congenita Mutation*

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The voltage-gated chloride channel CIC-1 is the major contributor of membrane conductance in skeletal muscle and has been associated with the inherited muscular disorder myotonia congenita. Here, we report a novel mutation identified in a recessive myotonia congenita family. This mutation, Gly-499 to Arg (G499R) is located in the putative transmembrane domain 10 of the CIC-1 protein. In contrast to normal CIC-1 channels that deactivate upon hyperpolarization, functional expression of G499R CIC-1 yielded a hyperpolarization-activated chloride current when measured in the presence of a high (134 mM) intracellular chloride concentration. Current was abolished when measured with a physiological chloride transmembrane gradient. Electrophysiological analysis of other Gly-499 mutants (G499K, G499Q, and G499E) suggests that the positive charge introduced by the G499R mutation may be responsible for this unique gating behavior. To further explore the function of domain 10, we mutated two charged residues near Gly-499 of CIC-1. Functional analyses of R496Q, R496Q/G499R, R496K, and E500Q mutant channels suggest that the charged residues in domain 10 are important for normal channel function. Study of these mutants may shed further light on the structure and voltage-gating of this channel.

Myotonia congenita (MC) is a genetic disorder caused by mutations in the skeletal muscle voltage-gated chloride channel (CIC-1). Clinical electrophysiology studies show repetitive electrical discharges (myotonic runs) of muscle in patients with this disease. Abnormal electrical discharge in MC is caused by low chloride conductance of the muscle membrane (1–6). Genetic studies have linked MC to CLCN1 (7–9), the major skeletal muscle voltage-gated chloride channel gene (10–12). To date, approximately 35 CLCN1 mutations have been identified in MC patients (8, 13–25).

The skeletal muscle chloride channel is a member of the CIC family of voltage-gated chloride channels. Each CIC-1 subunit is a 110-kDa protein composed of 13 putative transmembrane domains (26–28). Evidence supports at least two different transmembrane topologies for a single CIC-1 subunit (28, 29) that co-assemble as a homomultimeric channel (18, 27, 30, 31).

Unlike voltage-gated sodium and potassium channels, in which the voltage dependence is coupled to the movement of several charged residues located on a transmembrane segment of the protein (32), no similar domain is present in the CIC-1 subunit. There are two models to account for the voltage-dependent gating of CIC channels. According to one model, the voltage-dependent opening of the CIC channel depends on the binding of chloride ions to the channel and the movement of these ions through the conduction pathway (33–35). The second model proposed the existence of an intrinsic voltage sensor (36), in which bound anions interact with a voltage-dependent gate. However, in either model, the domains involved with voltage sensing or anion binding remain unclear. In contrast to WT CIC-1 channels that normally deactivate in response to hyperpolarization under physiologic conditions, one CIC-1 mutant (D136G) has been shown to activate upon hyperpolarization (36, 37). A similar behavior of activation with hyperpolarization was observed for WT channels when studied at low extracellular pH (38). The mechanism for this behavior is unknown.

In this study, a novel Gly-499 → Arg (G499R) mutation was identified in a recessive MC (Becker’s disease) patient. Functional analysis revealed that G499R CIC-1 channels activate upon hyperpolarization, similar to D136G CIC-1 channels. Site-directed mutagenesis at several sites in putative transmembrane domain 10 (D10) revealed the importance of electrostatic effects in this unique gating behavior and suggested an important role for D10 in the voltage-dependent gating of the CIC-1 channel.

EXPERIMENTAL PROCEDURES

Identification of Patients—A Polish kindred (Fig. 1A; father, mother, and son) was evaluated, and the son was diagnosed with Becker’s MC. The proband and his parents underwent a complete neurological exam, with particular attention directed toward the neuromuscular system, including a standard electromyographic examination. A blood sample was drawn from each member of the family for DNA extraction.

PCR Amplification of DNA from Study Subjects—Intronic sequence was used to design primers (19) that allowed amplification of CLCN1 exons from genomic DNA. The 10-μl PCR mixture contained 50 ng of genomic DNA, 6.7 μl of DH2 O, 0.6 μl of dNTP (125 μM of each deoxynucleoside triphosphate), 0.3 μl (25 pmol/μl) of forward and reverse primer, 1 μl of reaction buffer (500 mM KCl, 100 mM Tris-HCl, 1.5 mM MgCl2, 0.01% gelatin), 0.25 units of Taq DNA polymerase (5 units/μl), Perkin-Elmer Corp.), and 0.1 μl of [α-32P]dCTP (Amersham Pharmacia Biotech). The mixtures were initially denatured at 94 °C for 3 min followed by 30 cycles each of 30 s for denaturing at 94 °C, 30 s for annealing at temperature, and 30 s for extension at 72 °C.

Single Strand Conformation Polymorphism (SSCP) Analysis and Sequencing—SSCP was carried out using previously described methods (23). Briefly, the PCR products were diluted and denatured in 50 μl of
Mechanism of Inverted Activation of ClC-1 Channel

0.1% SDS/10 mM EDTA. After the addition of loading dye, the mixtures were heated at 94 °C for 3 min. The denatured mixture (2 μl) was separated by electrophoresis through a 5% nondenaturing polyacrylamide gel at 40 W for 3–7 h. The gels were run under the following two conditions: room temperature with glycerol, and 4 °C without glycerol. Gels were transferred to filter paper, dried on a vacuum slab dryer for 1 h at 85 °C, and then exposed to x-ray film at −20 °C for 12–24 h. Eluted DNA (10 μl) from the aberrant bands was reamplified using both the original PCR primers pair with additional M13 sequencing primer tails. Samples were purified by a centrifugation wash with a Centricron-100 column (Amicon, Millipore Corp., Bedford, MA) and then sequenced on an Applied Biosystems model 373A DNA sequencer using the dideoxy termination method.

Site-directed Mutagenesis—Two missense mutations, G499R and G482R (21), and seven point mutations (see under “Results”) were introduced into the pRc/CMV-CIC-1 vector by standard two-step PCR-based site-directed mutagenesis. All PCRs were conducted using Pfu DNA polymerase (Stratagene, La Jolla, CA) for high fidelity amplifications. Two fragments were amplified in the first step, using either primer pair A/B or pair C/D, which contained the desired mutations, and pCMV-CIC-1 as a template. In the second step, the two partial overlapping fragments were joined by annealing and extended by polymerization. These full-length templates were amplified using primer pair A/D. The final products were digested with the appropriate restriction enzyme (HindIII, Promega, Madison, WI and EcoRI, Stratagene)) and ligated into the pRc-CMV-CIC-1 vector. All PCRs were carried out in a DNA Engine Tetrad (M.J. Research, Waltham, MA). The inserts were sequenced to exclude any polymerase errors.

Cell Culture and Transfection—Human embryonic kidney (HEK)-293 cells (American Type Culture Collection, CRL 1573) were grown on Dulbecco’s modified Eagle’s medium; supplemented with penicillin, streptomycin, and 10% fetal calf serum (Life Technologies, Inc.); and maintain in 37 °C with 5% CO2. One day after the cultures were split, the calcium/phosphate precipitation technique (39) was used for cell transfection. About 0.4 μg of the appropriate pRc/CMV-CIC-1 plasmid DNA was used for each transfection in 35-mm culture dishes. To allow identification of transfected cells during patch clamp experiments, cells were co-transfected with green fluorescent protein gene plasmid cDNA (CLONTECH Inc., Palo Alto, CA). Approximately 15 h after transfection, cells were split again, and the medium was changed.

Electrophysiology—Currents were recorded from 36 to 48 h after transfection by the whole-cell configuration of the patch clamp technique (40) using an Axopatch 200 B amplifier (Axon Instruments Inc., Foster City, CA). Data were acquired on-line by a personal computer with pClamp software and a Digidata 1200 A/D interface (Axon Instruments Inc.). Currents were low pass filtered at 2 kHz and digitized at 10 kHz. The external solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 5 mM HEPES at pH 7.4. The standard internal pipette solution contained 130 mM NaCl, 2 mM MgCl2, 5 mM EGTA, and 10 mM HEPES at pH 7.4. A lower internal CaCl2 concentration was achieved by equimolar substitution with sodium glutamic acid. Recording pipettes were pulled from borosilicate glass (Kimax, Kimble Glass Inc. Vineland, NJ) by a Sutter P-87 puller (Novato, CA) and were filled with standard internal solution. The junction potential was adjusted to zero for each experiment, and series resistance was compensated 80–90% by analog circuitry.

Pulse Protocols and Data Analysis—The current-voltage (I-V) relationship for CIC-1 channels was determined from a holding potential of −35 mV. Currents were elicited by 180-ms test pulses ranging from −190 to +170 mV. Instantaneous currents were determined by extrapolation of fitted current traces to the beginning of each test potential. The isochronal I-V relationship was obtained by measuring current at the end of each test potential.

The voltage dependence of relative open probability (P) was obtained by measuring the instantaneous current, or the peak of tail current (I ) at −135 mV after 1.4-s prepulses ranging from −130 to +170 mV. Tail currents were fitted by a two-exponential function, and the peaks of tail currents obtained by extrapolation were plotted as a function of test voltage and then fitted to a Boltzmann function,

\[ I_V = I_o + \left( \frac{I_{max} - I_o}{1 + \exp(V_{1/2} - V/dx)} \right) \]

where \( I_{max} \) is the maximal current, \( I_o \) is a constant offset, \( V_{1/2} \) is the half-maximal activation voltage, and \( dx \) is the slope factor. The relative \( P \) was obtained by the normalization \( P = I/2I_{max} \)

Current deactivation was determined using a 2-pulse protocol. Cells were held at −35 mV, and then the voltage was stepped to +45 mV for 500 ms. Tail currents were measured during a subsequent 180-ms test pulse to potentials ranging from −135 to +45 mV and applied in 20-mV incremental steps. The time courses of current deactivation and activation were determined by fitting current traces with a two-exponential function, \( I(t) = A_1 \exp(-t/t_1) + A_2 \exp(-t/t_2) + C \), where \( t \) and \( A \) is the time constant and the amplitude of the slow component, respectively; \( t_2 \) and \( A_2 \) are the time constant and the amplitude of the fast component, respectively; and \( C \) is a constant. Data were analyzed with pClamp (Axon Instruments, Inc.), Origin (Microcal Software Inc., Northampton, MA), and SigmaStat (SPSS Inc., Chicago, IL) software. All data are shown as mean ± S.E. Statistical evaluation was performed using Student’s t test and two-way analysis of variance.

RESULTS

A Novel G499R Mutation—Two different aberrant conformers were observed in SSCP analysis from DNA samples in this MC kindred (Fig. 1A). DNA sequencing revealed both a novel C-1495-T (antisense strand) transition in exon 14 (Fig. 1B) and a previously identified nonsense mutation (C-2680-T) in exon 23 of the CLCN1 gene. When read in-frame, these mutations predict a Gly to Arg change at amino acid position 499 and an Arg to Stop change at amino acid position 594 in the CIC-1 protein, respectively. Additionally, both mutations were found in the son diagnosed with Becker’s MC (Fig. 1A). Two hundred unrelated normal alleles have been screened for this C-1495-T conformer by SSCP analysis, and no similar aberrant pattern has been observed (data not shown). According to the putative channel topology, this G499R mutation is located at the end of the putative transmembrane D10 segment (Fig. 2A). Sequence alignment shows that Gly-499 is highly conserved among the different CIC homologues (Fig. 2B).

Functional Expression of G499R in HEK-293 Cells—In order to understand the functional consequence of this novel CIC-1 mutation and its role in MC, we characterized the biophysical properties of the G499R CIC-1 channels expressed in HEK-293 cells. Currents recorded from HEK-293 cells expressing either WT CIC-1 or G499R CIC-1 are shown in Fig. 3. Membrane hyperpolarization (from +45 mV) of cells expressing WT CIC-1 channels elicited a large inward current that rapidly deactivated. At steady state, the maximum inward current saturated near −100 mV (Fig. 3A, left). Upon depolarization (from +135 mV), WT channels activated and reached saturation near +100 mV (Fig. 3A, right). In contrast, the G499R mutant channels activated upon hyperpolarization did not deactivate nor saturate, even at −220 mV (Fig. 3B, left). Unlike WT CIC-1 channels, mutant channels closed upon depolarization (Fig. 3B, right).

The amplitude of G499R CIC-1 current was smaller than

\[ \text{Mechanism of Inverted Activation of ClC-1 Channel} \]
that of WT ClC-1 channel (Fig. 4, A and B). For example, at 2130 mV, the mean instantaneous current amplitude for WT was 3.0 ± 0.7 nA, and for G499R was 0.4 ± 0.03 nA. G499R ClC-1 also exhibited more pronounced inward rectification than WT channels, and effects were notable in both instantaneous and isochronal I-V relationships (Fig. 4, C and D). In addition to the decrease in current amplitude, the voltage dependence of the relative open probability ($P_o$) of G499R ClC-1 channels was dramatically altered. In the voltage range from 2200 to 1100 mV, the $V_{1/2}$ for WT ClC-1 could be described by a single Boltzmann function ($V_{1/2} = -64.1 \pm 3.0$ mV; slope factor, 24.3 ± 0.8 mV). In contrast to WT, the voltage dependence of $P_o$ for G499R ClC-1 channels was completely reversed (Fig. 4, E). In the voltage range from 2200 to 150 mV, the $V_{1/2}$ was -56.6 ± 2.4 mV, with a slope factor of -22.2 ± 2.2 mV.

To further investigate the pathogenic role of the G499R mutant channel in MC, we also studied its function using a physiological transmembrane gradient of chloride concentration. Current traces recorded with 5 mM intracellular (pipette) chloride from both the WT ClC-1 and the G499R ClC-1 channels are shown in Fig. 5, A and B. The G499R mutation caused a dramatic reduction in chloride conductance (Fig. 5, C and D). For example, at 2105 mV, the average instantaneous current amplitude for WT ClC-1 was 0.7 ± 0.1 nA, but it was only 0.01 ± 0.004 nA for the G499R mutant. The dramatic reduction of G499R current at a physiological chloride concentration fully explains its pathogenic role in MC.

**Functional Analysis of G499K, G499Q, and G499E Mutations**—In order to understand how the G499R mutation causes such a dramatic change in the gating of the ClC-1 channels, we constructed several missense mutations of Gly-499 in the ClC-1 protein. First, to investigate whether or not Arg is necessary for this unique voltage-dependent gating, we replaced the Gly at
position 499 with Lys, which is similar to Arg in both size and charge. If the unusual gating of G499R ClC-1 channels was only charge-related, then the properties of G499K should be similar to G499R. Fig. 6, A and B, shows current traces recorded from HEK-293 cells transfected with G499K ClC-1 cDNA. As predicted, these currents were similar to G499R ClC-1 (Fig. 3, A and B). The \( P_n \) voltage curve for G499K ClC-1 was also similar to G499R ClC-1 (Fig. 6C, \( V_{1/2} = -56.9 \pm 4.6 \) mV; slope factor, \(-17.4 \pm 2.3 \) mV; for G499K). In the voltage range of \(-130 \) to \(+50 \) mV, the \( V_{1/2} \) of the \( P_n \) voltage curve was the same as that for G499R (\( p > 0.05 \)). This hyperpolarization-activated gating of the G499R and G499K mutants could result from either electrostatic or structure changes (or both) at this residue.

To distinguish among these possibilities, we then introduced a Gln at position 499. This residue is similar in size to Arg and Lys but neutral in charge. The gating of G499Q ClC-1 channels (Fig. 7, A and B) was similar to WT ClC-1 channels (Fig. 3A), suggesting that the hyperpolarization activation is an electrostatic effect of the G499R mutant. However, unlike WT ClC-1 channels, the \( P_n \) voltage curve of G499Q ClC-1 channel currents could not be simply fitted by single Boltzmann function (Fig. 7C).

To further evaluate the importance of the electrostatic forces at this position, we mutated this residue to Glu, which is similar in size to Arg but negatively charged. The G499E channels did not exhibit the inverted-gating property of G499R (Fig. 4). Instead, G499E ClC-1 channels deactivated slowly upon hyperpolarization from a holding potential of \(-130 \) mV to the test potential (\(-135 \) mV), the tail current remained constant (\( P_n = 0.3 \)) even though the conductance (\( g_{\text{max}} \)) decreased throughout this range. That is, conductance decreased with decreasing driving force, but channel gating was not voltage-dependent in this range of potentials (Fig. 8D). The \( P_n \) of G499E increased over the range of positive potentials and could be described by a single Boltzmann function with a \( V_{1/2} \) of \( 89.7 \pm 4.9 \) mV and a slope factor of \( 48.2 \pm 3.2 \) mV (Fig. 8D). These data indicate that substituting a negative residue for Gly-499 not only abolished the inverted voltage-dependent gating property of G499R but also impaired the closing of ClC-1 channels at negative potentials.

**Functional Consequences of R496Q, R496Q/G499R, R496K, E500Q, and G482R Mutations in ClC-1 Channels**—Due to the importance of charged residues at position 499 in D10, we also examined Arg-496, the only charged residue in D10 of the WT channel (Fig. 2). Expression of channels with Arg-499 changed to a neutral residue (Gln) did not yield any detectable chloride current (data not shown). Because Arg-496 and Gly-499 are in close proximity in the putative a-helical structure for D10, we hypothesized that G499R might compensate for the charge loss of the R496Q mutation. However, the double mutant (R496Q/ G499R) did not functionally express (data not shown). We replaced Arg-496 with Lys (R496K) and showed that channel current was similar to that of WT (Fig. 9, A and B). However, the \( P_n \) voltage curve was shifted to more negative potentials when compared with WT (Fig. 9C, \( V_{1/2} = -97.8 \pm 5.2 \) mV; \( p < 0.05 \)). Taken together, these results suggest that a positive charge at position 498 is essential for normal channel function.

Another naturally occurring MC mutation is known to introduce an additional charged residue in D10. The G482R mutation was originally identified in a Becker’s MC family (21) (Fig. 2). As with the R496Q mutant, expression of G482R ClC-1 channels did not yield detectable current (data not shown).

To further investigate the importance of charge in this region of ClC-1, we also mutated Glu-500, the first charged residue in the putative loop between D10 and D11 (Fig. 2), to Gln (E500Q). Expression of E500Q ClC-1 channels yielded a current similar to WT (Fig. 10, A and B) without having significant effects on the voltage dependence of \( P_n \) (\( p > 0.05 \); Fig. 10C).
Mechanism of Inverted Activation of ClC-1 Channel

Fig. 7. G499Q ClC-1 channel currents. Currents were recorded from HEK-293 cells transfected with G499Q ClC-1 cDNA, using deactivating (A) and activating (B) protocols. C, voltage dependence for relative $P_o$ of G499Q ClC-1 channels (filled squares) ($n = 6$). The $P_o$ was obtained by connecting data points with a smooth line. The $P_o$ curve for wild-type is shown by the dashed line (from Fig. 4E).

These data indicate that electrostatic effects due to charge alterations at position 500 are not as important for ClC-1 function as changes at position 482, 496, or 499.

DISCUSSION

Mechanism of MC Caused by G499R—MC has been recognized for over a century (41). The muscle stiffness and delayed relaxation observed in MC are the result of repetitive electrical discharges and hypereexcitability of muscles that is known to result from decreased Cl$^-$ conductance of sarcolemma (1–6). However, elucidation of the molecular basis of this disease was not possible until the cloning of the ClC-1 gene. Although mutational analysis and functional characterization of ClC-1 channels have provided some insights into MC, the mechanisms of disease need to be further defined and may shed light on the function of WT channels.

In the present study, we identified a novel G499R mutation and a previously known nonsense R894X mutation (14, 23) in a Becker’s MC family. Our genetic analysis indicates that G499R and R894X are transmitted as recessive alleles. Under physiological ionic conditions, the conductance of the G499R ClC-1 channel was undetectable. Thus, G499R ClC-1 channels are unlikely to contribute any chloride conductance in muscle membranes. A similar phenomenon has also been described for the D136G mutation (36). Despite the fact that R894X mutation produces a truncated protein, expression of mutant channels was reported to cause a reduction but not a complete abolition of chloride conductance (21). Therefore, it is likely that any muscle membrane chloride conductance in our patient results from channels encoded by the R894X allele.

Implication of G499(R/K) for a Model of Inverted ClC-1 Gating—Hyperpolarization-activated gating behavior of ClC-1 observed in the G499(R/K) mutants has been noted previously in a naturally occurring Becker’s mutation, D136G (36), and in WT ClC-1 channels when external pH was lowered to 5.5 (38).

In neither case is the factor responsible for this unique voltage-dependent gating known. We have demonstrated that the introduction of a positive charge at position 499 (G499R or G499K) of D10 could also evoke this unique gating of ClC-1. It has been proposed that D136G, located in D1, could be involved in voltage-dependent gating of ClC-1 channels (36). Similar gating defects caused by both G499R and D136G mutations suggest that charge changes at these positions of ClC-1 could affect voltage-dependent gating in a similar way in these mutant channels.

The mechanism underlying the voltage-dependent gating of ClC-1 channels remains unclear. However, it has been proposed that Cl$^-$ may act as a gating charge in the depolarization-activated gating of ClC channels (33–35). According to this model, WT channels have Cl$^-$ binding sites that are sensitive to external Cl$^-$ concentrations. The voltage dependence of gating is therefore determined by the binding of Cl$^-$ to these sites and the movement of Cl$^-$ inside the conducting pore. Alternatively, Cl$^-$ also may interact with an intrinsic voltage sensor, in which the gating of the ClC-1 channel could be affected by anion binding to the sites lying along the pore pathway (36, 42, 43). In these models, the voltage dependence of opening in ClC-1 is dependent upon the binding of Cl$^-$ to these sites. Although evidence supports the role of Cl$^-$ in voltage-dependent gating, more studies are needed to identify the putative Cl$^-$ binding site. It is possible that the charge reversal in D10 (or D1) affects the efficiency of anion binding, which in turn alters open probability of the channel.

Mutation of Gly-499 to a positively charged residue (Arg or Lys) inverted the voltage dependence of gating of ClC-1 such that $P_o$ was maximal at very negative and positive transmembrane potentials, with a minimum value near 0 mV. These two phases of the $P_o$-voltage curve suggests the existence of two
different gating mechanisms in these mutant channels. To explain this, we propose a model based on the chloride-dependent mechanisms of gating described above. Because the inverted gating behavior was only observed for the G499R and G499K mutants, we propose that an additional positively charged residue in D10 may introduce a new chloride binding site near the conducting pore of the ClC-1 channel. This new binding site could be occupied by intracellular Cl\(^{-}\) rather than extracellular Cl\(^{-}\) in a voltage-dependent manner. Thus, hyperpolarization to a negative transmembrane potential favors the binding of intracellular Cl\(^{-}\) at this new site rather than to the electrostatic forces, which leads to an increase of channel open probability (Figs. 4E and 6C). Upon depolarization to positive transmembrane potentials, the chloride binding is favored from the outside, and the \(P_o\) would start to increase (Figs. 4E and 6C). Previous findings indicated that the \(P_o\) and kinetics of ClC-1 channels depend on external but not internal Cl\(^{-}\) concentration (38). In contrast, the putative new binding site makes the G499(R/K) ClC-1 channels more sensitive to intracellular Cl\(^{-}\) concentration than WT channels. A low intracellular Cl\(^{-}\) concentration (e.g. 5 mM) will not favor internal Cl\(^{-}\) binding and therefore will cause a decrease of inward current (Fig. 5). According to this model, the descending phase of the \(P_o\)-voltage relationship for G499(R/K) ClC-1 channels at negative membrane potentials is due to a gating mode modulated by the new Cl\(^{-}\) binding site. In contrast, the ascending phase of \(P_o\) in these mutant channels at positive membrane potentials likely represents normal chloride gating involving the normal anion binding sites mechanism, which is described in WT ClC-1 channels. Both G499R and D136G mutations result in the addition of positive charge or reduction of negative charge. Very likely, as in G499R ClC-1 channels, the charge change plays a similar role in the hyperpolarization-activated D136G ClC-1 channels.

The anion-dependent gating mechanism could also be applied to the G499E mutant channels. Concerning the importance of the electrical charge on D10, the additional negative charge at position 499 could disrupt the normal chloride-binding sites of WT ClC-1 channels, or at least electrostatically repulse the presumed gating charge and disrupt Cl\(^{-}\) binding to the extracellular sites. In this case, the channel opening is not voltage-dependent at negative potentials (Fig. 6D). However, the disruption of Cl\(^{-}\) binding may be compensated by positive transmembrane potentials in a voltage-dependent manner. As the membrane becomes more depolarized, binding of Cl\(^{-}\) to the external binding site increases, and the open probability of G499E channels increases (Fig. 6D). Falhke et al. (44) have proposed the existence of a negatively charged cytoplasmic gate to explain the deactivation of ClC-1 channels. The electrostatic repulsion between Glu-499 and this putative gate also may affect the kinetics of ClC-1 channels, in which markedly slower kinetics were observed (Fig. 8C). Our observations on G499R, G499K, and G499E ClC-1 channels further support the role of chloride ions in the voltage-dependent gating of this channel.

One may argue an alternative explanation for the biphasic \(P_o\)-voltage relationship for G499(R/K) channels based on the double-barreled model of ClC-1 channels. For example, a G499R homodimer containing one normal pore and a second pore that is affected by the mutation could have a descending \(P_o\)-voltage relationship (due to the mutant pore) and an ascending \(P_o\)-voltage relationship (due to the WT pore). The sum of two such processes could give the biphasic relationship that we observed. However, recent experimental data on ClC-0 (27, 45) and ClC-1 (45) suggest that functional voltage-gated chloride channels are formed as homodimers, in which each pore is very likely formed by a single subunit instead of different portions of both subunits. These data suggest that different contributions.
by two barres is an unlikely explanation for the biphasic $P_o$-voltage relationship observed in vitro for homodimeric mutant channels. However, controversy still exists regarding the single- versus double-barrel structure of CIC-1 channels.

**Implication of D10 for CIC-1 Gating**—The Arg at position 496 is the only charged residue in D10 and is highly conserved among all CIC channels (Fig. 2). Similar to R496Q, the naturally occurring mutation R496S also did not yield any detectable current (17). Furthermore, the G499R mutation failed to compensate for the R496Q charge loss, although they are able current (17). Moreover, the G499R mutation failed to among all ClC channels (Fig. 2). Similar to R496Q, the natural Arg at position 496 is critical for normal channel function. We cannot exclude the possibility that the positively charged residue in D10 (R496) interacts with normal anion-binding sites or alters gating by an allosteric mechanism via interaction with another domain in the channel. The neutralized Gln might impair or weaken this binding site and, therefore, produce a nonconducting channel. Alternatively, we also cannot exclude the possibility that Arg-496 is important as a part of an intrinsic voltage-sensor in WT CIC-1 channels, similar to the mechanism of D136G proposed by Fahkle et al. (36, 44). In this scenario, the replacement with Gln at this position would disrupt the voltage-sensor, resulting in a nonfunctional channel. Interestingly, the replacement of Arg-496 with Lys favors channel opening (Fig. 9C). The parallel leftward shift of the $P_o$-voltage relationship further suggests that this charged residue in D10 is involved in the voltage-dependent gating of CIC-1 channels. Surprisingly, we did not observe any significant effect when the highly conserved negatively charged Glu at position 500 was replaced with Gln. This implies that this charged residue, located in the loop between D10 and D11, is not as critical as those in D10 for the voltage-dependent gating of CIC-1 channels. Finally, the G482R mutation, located at the amino-terminal end of D10, completely abolishes chloride conductance. In contrast to the G499R mutation, in which the channels are still conducting, this observation suggests that the G482R may also affect folding, trafficking or degradation of the CIC-1 channels.

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