Involvement of Helices at the Dimer Interface in ClC-1 Common Gating

Michael Duffield,[1] Grigori Rychkov,[1,2] Allan Bretag,[2] and Michael Roberts[1]

[1]Department of Physiology, University of Adelaide, Adelaide, South Australia 5005, Australia
[2]Centre for Advanced Biomedical Studies, University of South Australia, Adelaide, South Australia 5000, Australia

ABSTRACT ClC-1 is a dimeric, double-pored chloride channel that is present in skeletal muscle. Mutations of this channel can result in the condition myotonia, a muscle disorder involving increased muscle stiffness. It has been shown that the dominant form of myotonia often results from mutations that affect the so-called slow, or common, gating process of the ClC-1 channel. Mutations causing dominant myotonia are seen to cluster at the interface of the ClC-1 channel monomers. This study has investigated the role of the H, I, P, and Q helices, which lie on this interface, as well as the G helix, which is situated immediately behind the H and I helices, on ClC-1 gating. 11 mutant ClC-1 channels (T268M, C277S, C278S, S289A, T310M, S312A, V321S, T539A, S541A, M559T, and S572V) were produced using site-directed mutagenesis, and gating properties of these channels were investigated using electrophysiological techniques. Six of the seven mutations in G, H, and I, and two of the four mutations in P and Q, caused shifts of the ClC-1 open probability. In the majority of cases this was due to alterations in the common gating process, with only three of the mutants displaying any change in fast gating. Many of the mutant channels also showed alterations in the kinetics of the common gating process, particularly at positive potentials. The changes observed in common gating were caused by changes in the opening rate (e.g. T310M), the closing rate (e.g. C277S), or both rates. These results indicate that mutations in the helices forming the dimer interface are able to alter the ClC-1 common gating process by changing the energy of the open and/or closed channel states, and hence altering transition rates between these states.

KEY WORDS: chloride channel • mutation • patch-clamping • myotonia

INTRODUCTION

Chloride channels of the ClC family have many physiological roles, however, compared with cation channels of similar importance, little is known about the relationships between structure and function of channels in this family (see Jentsch et al., 2002). The X-ray crystallographic images of a bacterial ClC protein recently obtained show a structure vastly different from that of other ion channels (Dutzler et al., 2002). As earlier electrophysiological studies had proposed, the ClC protein is a homodimer with each subunit containing its own pore (Middleton et al., 1994, 1996; Ludewig et al., 1996; Saviane et al., 1999). The crystal structure shows that each subunit of ClC contains 18 α-helical regions, and those that lie within the membrane are of variable length and often oblique, with most not spanning the whole membrane (Dutzler et al., 2002). While the crystal structure allows identification of the amino acid residues lining the conduction pathways and forming the selectivity filter of the channel it does not definitively identify the regions of the channel involved in gating.

Considerable information about the gating process of two members of the ClC family, ClC-0 from the electroplax of *Torpedo*, and ClC-1 from mammalian skeletal muscle, has been gleaned from electrophysiological studies (for review see Jentsch et al., 2002). Both of these channels have two different gating processes; a fast gating process in which individual pores open and close independently of one another ("fast gates"), and a slower gating process that closes both pores simultaneously ("slow gate" or "common gate"). Gating of these members of the ClC family appears to be voltage dependent, but unlike the voltage-gated cation channels, ClC channels do not contain a similar region with charged residues that could form a voltage sensor. Instead, the voltage dependence arises from interaction of the permeating anion, usually chloride, with the gating site (Pusch et al., 1995a; Chen and Miller, 1996; Rychkov et al., 1996, 1998).

The physiological role of ClC-1 is to stabilize the resting membrane potential of skeletal muscle (Bretag, 1987). Mutations of this channel can reduce whole-cell Cl^- conductance, resulting in myotonia, a muscle stiffness disorder characterized by repetitive action potential firing, and prolonged muscle contraction (e.g., Pusch, 2002). Such myotonias can be inherited in both recessive and dominant forms. This varied inheritance pattern appears to result from differential effects of various mutations on the channel dimer (Kubisch et al., 1998; Saviane et al., 1999). In heterozygous individuals 75% of the dimeric channels will contain either one or two copies of the normal subunit. Mutations causing re-

Address correspondence to Grigori Rychkov, Department of Physiology, University of Adelaide, Adelaide, South Australia 5005, Australia. Fax: (61) 8 8303 3356; E-mail: grigori.rychkov@adelaide.edu.au

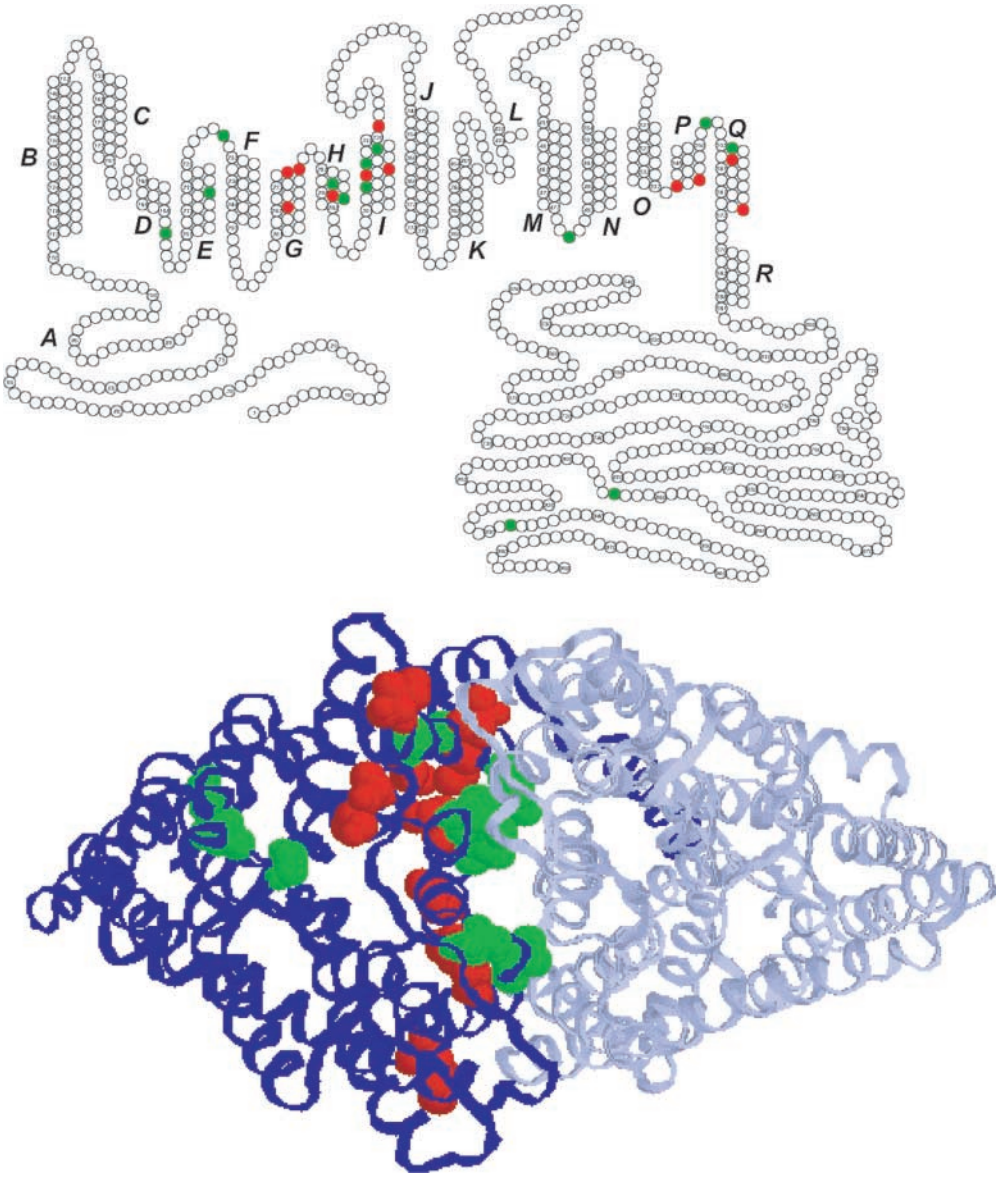


FIGURE 1. Structure and topology of the ClC channel. (A) Transmembrane topology of the ClC-1 channel, based on ClC structure determined by Dutzler et al. (2002), showing the transmembrane α-helical regions of the channel. (B) Crystal structure of the ClC dimer (adapted from Dutzler et al., 2002). In both figures the positions of naturally occurring mutations causing dominant myotonia are indicated in green, while the positions of the mutations investigated in the present study are indicated in red.

cessive myotonia most likely affect properties of the pore in one subunit, such as fast gating or conductance, leaving the other pore unaffected in WT/mutant heterodimers (Wollnik et al., 1997; Kubisch et al., 1998; Saviane et al., 1999; Weinreich and Jentsch, 2001). This would result in a residual whole cell Cl^- conductance of 50% or more, which is considered to be sufficient to avoid myotonic symptoms (Furman and Barchi, 1978). On the other hand, mutations causing dominant myotonia must affect both ClC-1 subunits to reduce the whole-cell Cl^- conductance in a heterozygous person to <50% in order to produce myotonic symptoms (Kwiecinski et al., 1988). The necessary interaction between the product of the mutated gene and that of the subunit transcribed from the WT gene is probably occurring through effects on the ClC-1 common gate, which is shared by both pores. A number of naturally occurring dominant negative ClC-1 mutations have been shown to alter the voltage dependence of the common gate (Saviane et al., 1999; Aromataris et al., 2001).

While the naturally occurring mutations in the ClC-1 channel that produce myotonia are scattered throughout the channel primary sequence, including the carboxyl tail, there is a cluster of dominant mutations at the interface of the channel monomers (Fig. 1), particularly in the H and I helices, although there are also some in the P and Q helices. Additionally, the mutations C212S and C213S in ClC-0, and C277S in ClC-1, which are in the G helix, affect the common gate (Lin et al., 1999; Accardi et al., 2001). This suggests that the G, H, I, P, and Q helices are important for the ClC common gating process. We have now investigated the effects of a further eleven point-mutations within these

helices on gating properties of the ClC-1 channel, and have shown that the majority of these mutant channels exhibit alterations in channel gating, particularly in the common gating process.

MATERIALS AND METHODS

Mutations

Eleven point mutations in the G, H, I, P and Q helices of the hClC-1 channel were studied in the present work. These mutations were: T268M, C277S, and C278S in helix G; S289A in helix H; T310M, S312A and V321S in helix I; T539A and S541A in the P helix; and M559T and S572V in helix Q. T268M was the only mutation investigated that has been reported previously as myotonic (Brugnoni et al., 1999). The C277S and C278S mutations are equivalent to the C212S and C213S mutations in ClC-0, which have been implicated in the common gating process of that channel. The remaining mutations were selected randomly to cover the G, H, I, P and Q helices, but with particular interest in residues in close proximity to known myotonic mutations. In these cases hydrophobic residues were switched for hydrophilic residues of about the same relative size, and vice versa.

Site-directed Mutagenesis

Two-step PCR-based site-directed mutagenesis (Ho et al., 1989) was used to introduce point mutations into hClC-1 cDNA (Steinmeyer et al., 1994). All PCRs used *Pwo* DNA polymerase (Roche Molecular Biochemicals) for high fidelity amplification. In the first step two fragments were amplified using primers containing the desired mutation and hClC-1 in the mammalian expression vector pCIneo (Promega) as a template. Recombinant PCR was then used to join the two fragments. The mutation-containing fragment was isolated using appropriate restriction endonucleases, and ligated into the pCIneo/hClC-1 vector. All PCR-derived fragments were completely sequenced to exclude polymerase errors.

Cell Culture and Transfection

Human embryonic kidney (HEK293) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% (vol/vol) fetal bovine serum (Trace), supplemented with L-glutamine (2 mM; Sigma-Aldrich), and maintained at 37°C in 5% CO_2. Cell cultures were transfected with 700 ng of either WT or mutant pCIneo/hClC-1 cDNA using LipofectAMINE PLUS reagent (Invitrogen), following the standard protocol described by the manufacturer, in 25-mm culture wells. Cells were cotransfected with 70 ng of green fluorescent protein plasmid cDNA (pEGFP-N1; CLONTECH Laboratories, Inc.), to allow identification of transfected cells during patch-clamp experiments. Cells were replated for patch-clamping at least 3 h after transfection, and electrophysiological measurements were commenced ~24 h after transfection.

Electrophysiology

Patch-clamping experiments were performed on transfected HEK293 cells in the whole-cell configuration using a List EPC 7 (List) patch-clamp amplifier and associated standard equipment, at room temperature (24 ± 1°C). Standard bath solution contained: 140 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, adjusted to pH 7.4 with NaOH. Standard pipette solution contained: 75 mM Cs glutamate, 40 mM CsCl, 10 mM EGTA, 10 mM HEPES, adjusted to pH 7.2 with KOH. Patch pipettes of 1–3 MΩ were pulled from borosilicate glass. Series resistance did not exceed 5 MΩ, and was 70–85% compensated. Currents obtained were filtered at 3 kHz, and collected and analyzed using pClamp software (Axon Instruments, Inc.). Potentials listed are pipette potentials expressed as intracellular potentials relative to outside zero. Data presented in figures and tables have been corrected for liquid junctions potentials, estimated using JPCalc (Barry, 1994).

Data Analysis

To approximate the time course of the ClC-1 current relaxations, raw current traces were fitted with an equation of the form:

$$I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C, \quad (1)$$

where A_1 and A_2 represent the amplitude of the fast and slow exponential components, τ_1 and τ_2 are their time constants, C represents the amplitude of the steady-state component, and t is time.

Overall apparent open probability (P_o) for WT and mutant channels was calculated from normalized peak tail currents for voltage steps to −100 mV after test pulses ranging from 120 to −140 mV. Data points were fitted with a Boltzmann distribution with an offset to obtain P_o curves:

$$P_o(V) = P_{min} + \frac{1 - P_{min}}{1 + \exp[(V_{1/2} - V)/k]}, \quad (2)$$

where P_{min} is an offset, or a minimum P_o at very negative potentials, V is the membrane potential, $V_{1/2}$ is the half-maximal activation potential, and k is the slope factor. Such a distribution assumes a maximal P_o of 1.

Open probability of the common gate was determined from protocols similar to those used to determine overall P_o, but with the addition of a brief (400 μs) activation pulse to 180 mV between the test pulse and the −100 mV tail pulse, to fully open the channel fast gates (Accardi and Pusch, 2000). Data points were fitted with Boltzmann distributions, as per Eq. 2.

Open probability of the fast gate was determined from the amplitudes of the fast deactivating component (Eq. 1) of ClC-1 currents in response to voltage steps ranging between −140 and −40 mV as described previously by Aromataris et al. (2001), or by dividing overall P_o by the P_o of the common gate (as described in Accardi and Pusch, 2000). Both methods gave similar results.

The opening rate (α) and closing rate (β) of the common gate at a particular voltage were calculated according to:

$$\alpha = P_o^c/\tau_2 \quad (3a)$$

$$\beta = (1 - P_o^c)/\tau_2, \quad (3b)$$

where τ_2 is the time constant of the slower gating relaxations (Eq. 1) and P_o^c is the open probability of the common gate.

RESULTS

All of the ClC-1 point mutants investigated (T268M, C277S, C278S, S289A, T310M, S312A, V321S, T539A, S541A, M559T, and S572V) produced currents deactivating at negative potentials with a double exponential time course (Fig. 2). The time constants of the exponential components of the currents of nine mutant

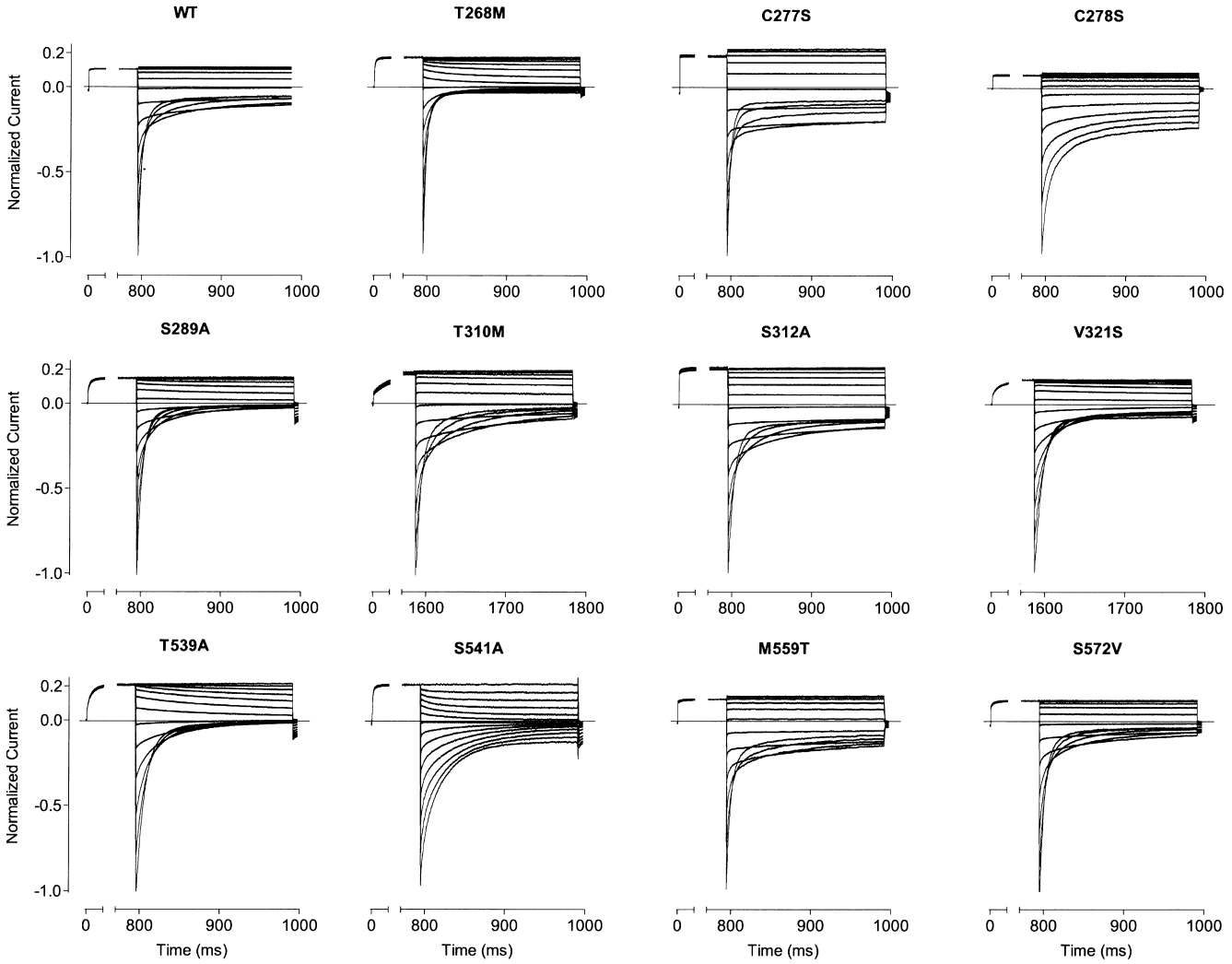


FIGURE 2. Whole-cell currents in HEK 293 cells expressing wild-type (WT) and mutant ClC-1 channels. Currents were activated by the following voltage protocol: holding potential was −30 mV; a prepulse to either 40 (WT, C277S, and C278S) or 100 mV (all other mutations) was followed by steps ranged from −140 to 120 mV in 20-mV increments. Currents in each panel are normalized to the maximum peak current observed at −140 mV.

channels out of eleven were all within 2.5-fold of those of the WT channel (Fig. 3), whereas S312A and M559T showed currents virtually identical to that of WT ClC-1 (not depicted). Visible changes of the kinetics of current deactivation were predominantly due to significant changes in the relative amplitudes of those exponential and steady-state components that reflect changes in the P_o of the fast and common gates (Aromataris et al., 2001).

Investigation of the voltage dependence of the P_o of mutant channels in the G, H, and I domains showed that some mutations shifted $V_{1/2}$ to more positive potentials (T268M, S289A, T310M, and V321S), while others shifted $V_{1/2}$ to more negative potentials (C277S and C278S), and the mutation S312A had no effect on $V_{1/2}$ (Fig. 4, Table I). Separation of the P_o of the fast and the common gates by either of the methods described previously (Accardi and Pusch, 2000; Aromataris et al., 2001) revealed that only two of the mutations investigated (T268M and C278S) affected the P_o of the fast gate (Fig. 5, Table I), while five mutations (T268M, C277S, S289A, T310M, V321S) significantly changed the P_o of the common gate (Fig. 5, Table I). The majority of mutations shifted the $V_{1/2}$ toward more positive potentials and reduced the minimum P_o. Mutations C278S and C277S, however, did not shift the $V_{1/2}$, but increased the minimum P_o of the fast and common gates, respectively.

Mutations in the P and Q domains also showed altered voltage dependence of gating, with three out of the four mutations investigated (T539A, S541A, and S572V) shifting $V_{1/2}$ of the P_o of the common gate to

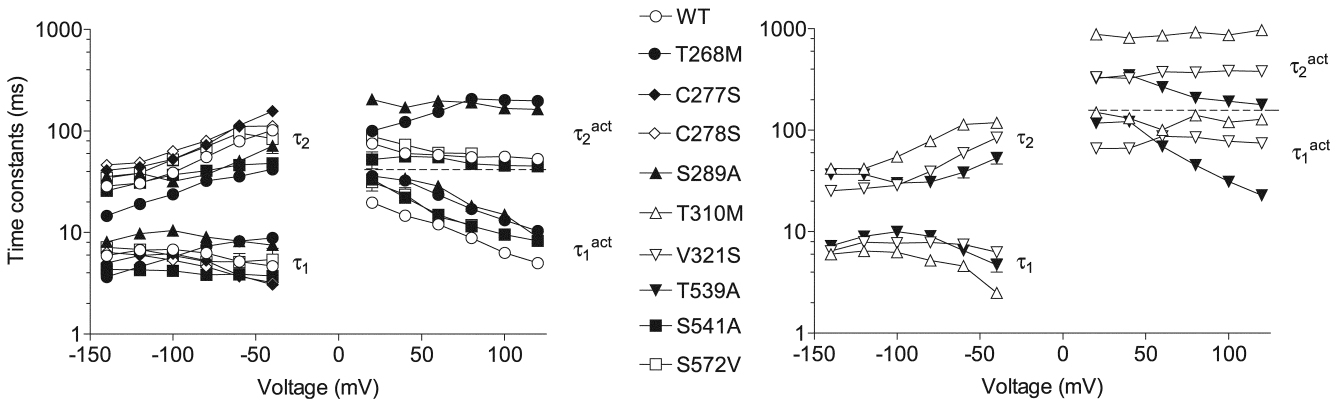


FIGURE 3. Time constants of current relaxations of ClC-1 WT and mutant channels. Time constants at negative potentials are extracted from deactivating currents, as shown in Fig. 2, and correspond to the relaxation of the fast (τ_1) and common (τ_2) gate. Time constants at positive potentials (τ_1^{act}, τ_2^{act}) are extracted from activating currents, as shown in Fig. 7, and correspond to relaxation of the common gate (n = 3–12).

more positive potentials, while S541A also showed a shift in fast gating (Fig. 5, Table I).

Some of the mutant channels investigated in this study (T268M, S289A, T310M, V321S, and T539A) required prepulses to 120 mV, for up to 1,600 ms, for full activation of the common gate. For better assessment of the changes produced by the mutations we therefore used a similar protocol for all channels, including WT and C277S channels. Comparison of the P_o curves of the C277S mutant (which has been shown to lock the ClC-1 common gate open; Accardi et al., 2001), obtained using protocols with either 200 or 800 ms long prepulses, shows that they are almost identical (Fig. 6, A and B) and therefore it is unlikely that the longer protocols used in the present study have introduced significant errors in determination of the $V_{1/2}$ or minimum P_o due to Cl^- accumulation or depletion. Results were also similar for the WT channel; however, with longer voltage protocols the overall P_o curves were somewhat shifted to the right, and minimum P_o of the common gate was lowered from 0.41 to 0.36 (Fig. 6, C and D), suggesting the presence of a small slower com-

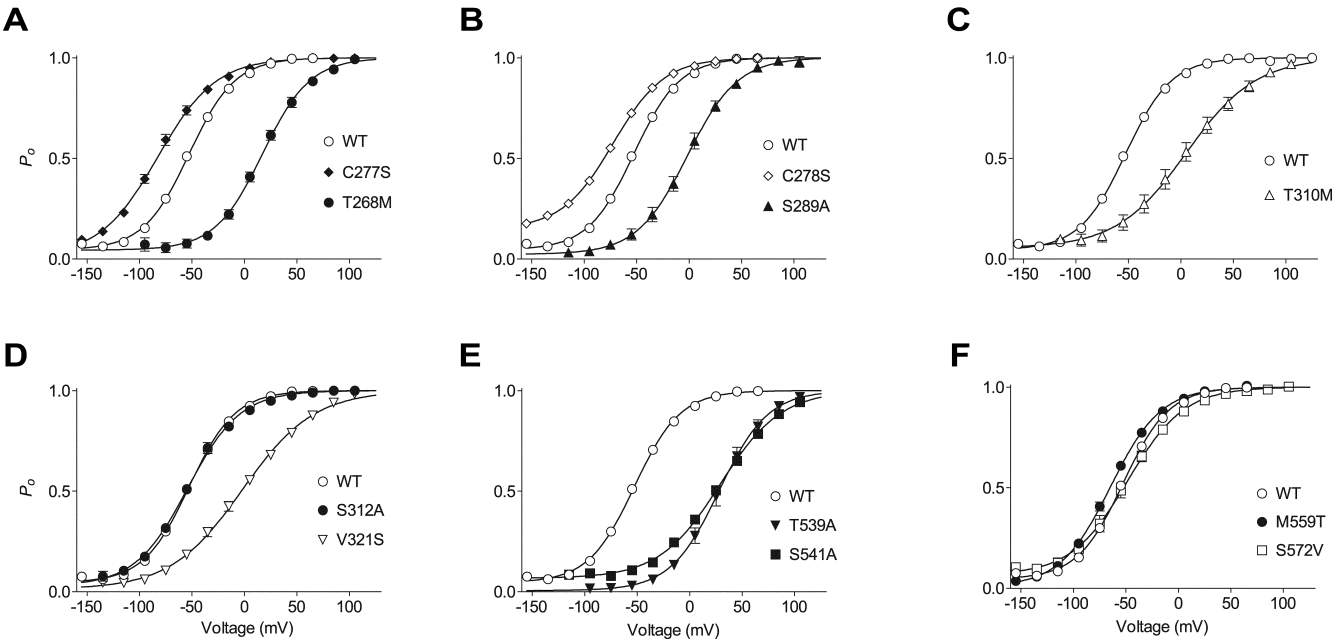


FIGURE 4. Open probability of WT and mutant ClC-1 channels as a function of membrane potential. Data points are extracted from tail currents as described in MATERIALS AND METHODS and fitted with a Boltzmann distribution with an offset (Eq. 2; n = 4–12).

TABLE I

Gating Parameters for WT and Mutant ClC-1 Channels

Mutant	Overall P_o		Fast P_o		Common P_o	
	$V_{1/2}$	P_{min}	$V_{1/2}$	P_{min}	$V_{1/2}$	P_{min}
	mV		*mV*		*mV*	
WT	−52.7 ± 1.0	0.04 ± 0.01	−107.3 ± 1.7	0.05 ± 0.02	−68.5 ± 1.2	0.36 ± 0.01
T268M	17.2 ± 1.0	0.04 ± 0.01	−45.7 ± 2.4	0.13 ± 0.02	16.4 ± 0.6	0.09 ± 0.09
C277S	−82.5 ± 1.5	0.02 ± 0.02	−101.8 ± 1.8	0.05 ± 0.03	−66.2 ± 4.9	0.72 ± 0.02
C278S	−72.2 ± 0.4	0.15 ± 0.01	−94.4 ± 0.6	0.40 ± 0.01	−72.3 ± 1.0	0.39 ± 0.01
V286A	Shifts by +61					
S289A	−1.7 ± 1.0	0.02 ± 0.01	−100.8 ± 2.9	0.02 ± 0.04	8.2 ± 1.0	0.05 ± 0.01
I290M	Shifts by +75		Shifts by +74		Shifts by +87	
F307S	Shifts by +74		Shifts by +14		Shifts by +63	
T310M	5.2 ± 1.3	0.05 ± 0.01	−109.1 ± 4.3	0.09 ± 0.05	11.0 ± 1.5	0.01 ± 0.01
S312A	−54.2 ± 1.2	0.03 ± 0.01	−111.4 ± 3.6	0.03 ± 0.05	−53.5 ± 2.0	0.33 ± 0.02
A313T	Shifts by +113		Shifts by +9		Shifts by +99	
V321S	−2.1 ± 1.4	0.01 ± 0.01	−102.2 ± 3.6	0.02 ± 0.04	5.9 ± 1.5	0.10 ± 0.01
T539A	28.1 ± 0.4	0.01 ± 0.01	−92.0 ± 2.7	0.03 ± 0.04	25.9 ± 0.2	0.05 ± 0.01
S541A	29.0 ± 0.9	0.07 ± 0.01	−41.7 ± 4.1	0.10 ± 0.05	29.8 ± 0.5	0.19 ± 0.01
M559T	−64.9 ± 0.6	0.01 ± 0.01	−121 ± 4.0	0.02 ± 0.06	−60.8 ± 1.0	0.20 ± 0.01
S572V	−48.6 ± 1.0	0.07 ± 0.01	−102.7 ± 4.9	0.08 ± 0.06	−47.3 ± 1.5	0.23 ± 0.01

Parameters of Boltzmann fits ($V_{1/2}$ and P_{min}) for the P_o of WT and mutant ClC-1 channels and the P_o of their fast and common gates (n = 3–10). Also shown are the changes in $V_{1/2}$ caused by dominant negative mutations previously investigated by others: V286A (Kubisch et al., 1998), I290M (Pusch et al., 1995b; Accardi and Pusch, 2000), F307S, and A313T (Aromataris et al., 2001).

ponent in ClC-1 gating, in addition to the fast and slow gating processes usually investigated (c.f. Rychkov et al., 1996).

Two exponential components of ClC-1 current relaxations reflect the fast and common gating processes and can be consistently extracted from deactivating macroscopic currents at negative potentials (Saviane et al., 1999). At more positive potentials, gating relaxations become exponentially faster, to such an extent that the time constant of the fast gate cannot be extracted from the currents at 80 mV, as it falls below 100 μs (Accardi and Pusch, 2000). Time constants of the common gate relaxation remain in the range of milliseconds for potentials of up to 200 mV and, theoretically, can be obtained from the time course of ClC-1 activation at positive potentials. We recorded activating ClC-1 currents in response to positive voltage steps, following a prepulse to −120 mV (Fig. 7). Analysis of the current kinetics revealed that WT ClC-1 activated with a double exponential time course, with time constants in the range of 5–20 ms and 50–100 ms (Fig. 3). The mutation C277S, which removed most of the common gating, also drastically diminished current activation at positive potentials, to the point where activating components could not be reliably extracted. In contrast, mutations T268M, S289A, T310M, V321S, and T539A, which shifted $V_{1/2}$ of the common gating to more positive potentials and reduced minimum P_o, also demonstrated increased current activation at positive potentials (Fig. 7). Activating currents for all of these mutants could be fitted with two exponential components. In the T310M, V321S, and T539A mutants these components were significantly slower than those of the WT, T268M, and S289A channels (Fig. 3). In the WT channel and most of the mutants the time constant of the major exponential component, which was the faster component that accounted for 70–90% of the current amplitude, showed clear voltage dependence similar to that reported by Accardi and Pusch (2000). In T310M and V321S mutants the major component was the slower one, and unconstrained double exponential fit produced time constants that were virtually voltage independent. Open probability of both these mutants, however, is clearly voltage dependent in this voltage range. Therefore it is likely that the voltage pulses that were employed to activate the currents were not long enough (1,600 ms) for accurate extraction of the slow time constants. Adequately long pulses to such high voltages, however, usually resulted in the electrical breakdown of the cell membrane and could not be routinely used.

At voltages where time constants could be extracted from activating or deactivating currents, and with the P_o known, it was possible to calculate opening and closing rates of the common gate of the WT and mutant channels, using Eqs. 3 a and 3 b. Calculations showed that mutation C277S reduced the closing rate of the common gate without affecting its opening rate. In contrast, T310M reduced the opening rate, without affecting the closing rate of the common gate. Other mu-

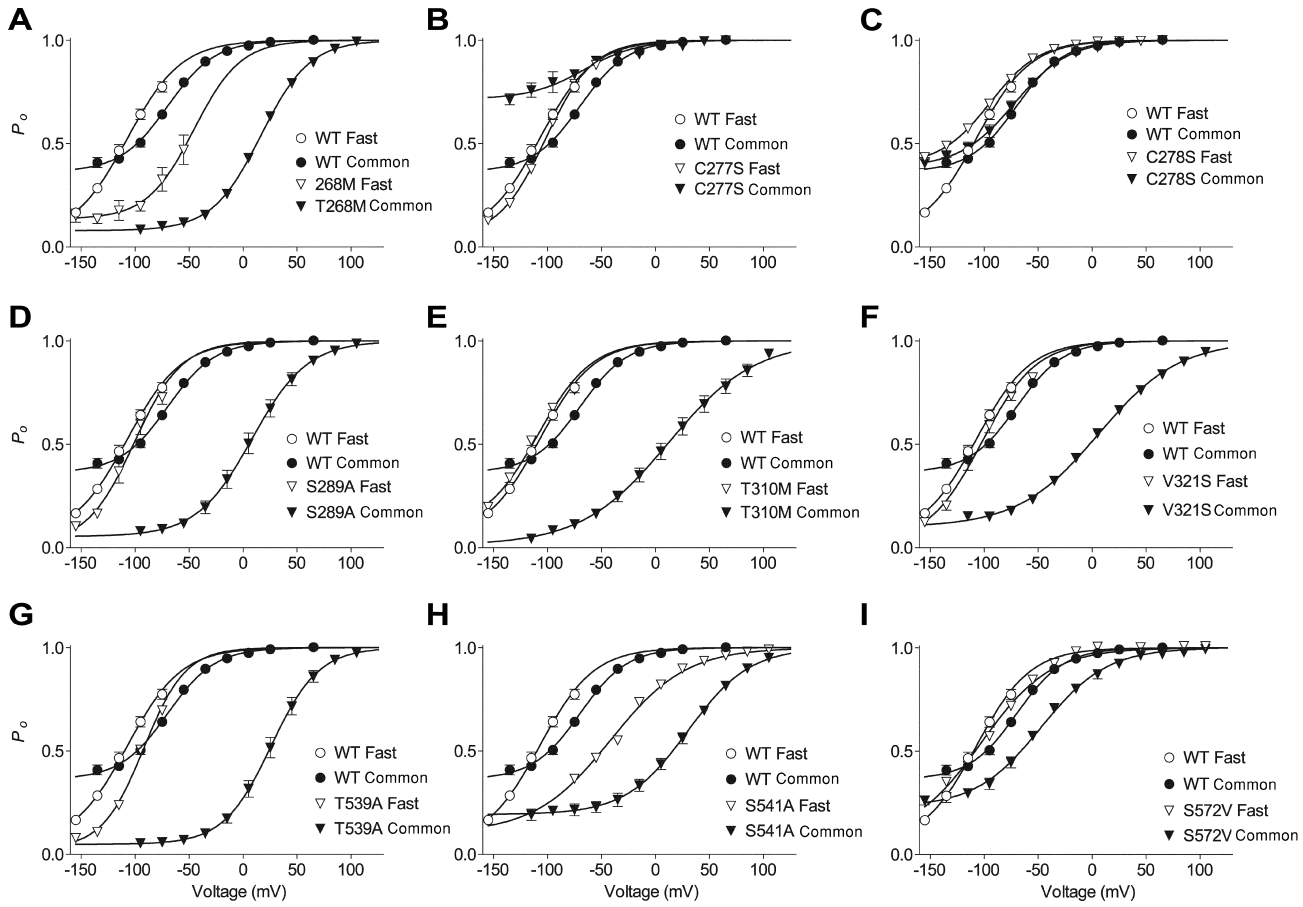


FIGURE 5. Open probability of the fast and common gate of WT and mutant ClC-1 channels as a function of membrane potential. Data points are obtained as described in MATERIALS AND METHODS and fitted with a Boltzmann distribution with an offset. (Eq. 2; $n = 4$–10).

tations affected both opening and closing rates to different extents (Fig. 8).

DISCUSSION

Results from single-channel recordings and extensive mutagenesis studies have long suggested that ClC-0 and ClC-1 channels have a double-barrelled structure with two parallel, independent pores (Ludewig et al., 1996; Middleton et al., 1994, 1996; Saviane et al., 1999). It has been proposed that each pore of these channels is gated by an independent, so-called "fast" gating process, while another process, the "slow" or "common" gating process, opens and closes both pores simultaneously. High-resolution crystal structures of two bacterial proteins homologous to ClC-1 have now provided a definite confirmation of the double-barrelled structure of ClC channels. The molecular mechanisms of gating in both ClC-1 and ClC-0 channels, however, remain poorly understood. In particular it is yet to be determined whether the common gate is a distinct molecular structure that closes both protopores simultaneously, or if the common gating process arises from an interaction between the two fast gates that locks them simultaneously into a closed conformation. In any case, the common gating process must involve interaction between the two channel subunits, and, judging by its high temperature dependence (Pusch et al., 1997; Bennetts et al., 2001), is accompanied by substantial structural rearrangements.

Allosteric interactions between the two subunits of ClC channels are likely to occur through the regions where they are joined. According to the crystal structure (Dutzler et al., 2002), the orientation of the two subunits is such that the H and I helices of one monomer are in close proximity to the P and Q helices of the other. Consequently the dimer interface comprises four helices on each side. Unlike the bacterial ClC channels, ClC-0 and ClC-1 have extensive cytoplasmic NH_2- and COOH-terminal regions, and the interaction of these may also contribute to the common gating process (Fong et al., 1998). Of the H and I

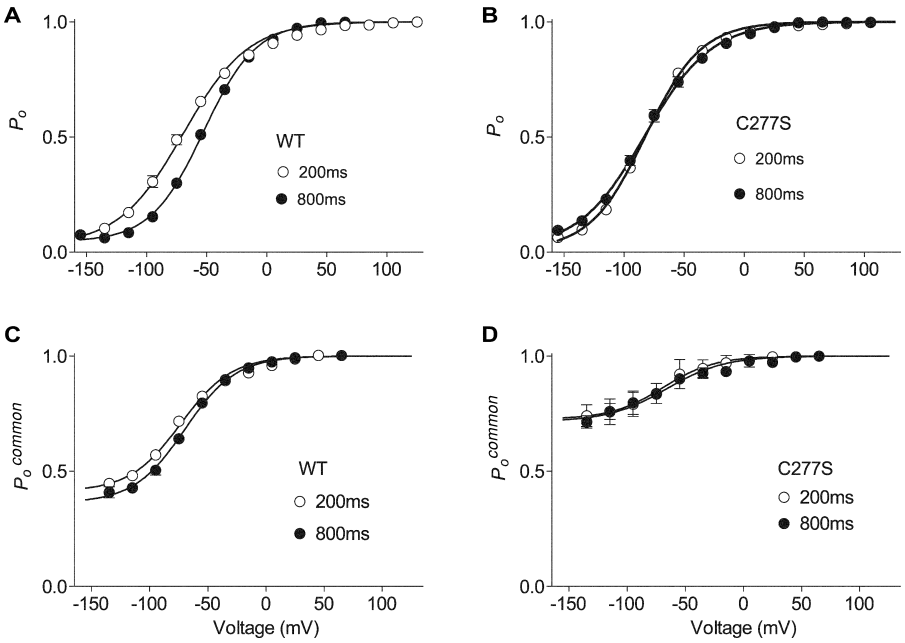


FIGURE 6. Effect of prepulse duration on P_o of WT and C277S ClC-1 channels. Overall P_o (A and B) and P_o of the common gate (C and D) of WT (A and C) and C277S (B and D) ClC-1 channels. Data points and curves are obtained as in Figs. 4 and 5 (n = 4–10).

helix mutations investigated in this study, three out of four altered the common gating mechanism, without changing fast gating. Two other known mutations in these helices, F307S and A313T, also affect only the common gating process (Aromataris et al., 2001; Table I). All of these mutations produced similar changes in the common gating process; $V_{1/2}$ shifted to more positive potentials, and minimum P_o was reduced to values close to zero. In addition, the current in these mutant channels activated much more slowly at positive potentials.

Mutations in the P and Q helices had similar effects to those in the H and I domains. Three out of four mutations investigated, two in the P helix and one the Q helix, caused shifts in $V_{1/2}$ to more positive potentials, and decreased minimum P_o of the common gate. Although the effect of the mutation M572V was not so dramatic, and mutation M559T showed the phenotype of the WT channel, at least one dominant myotonic mutation is currently known in the Q helix, I556N (Kubisch et al., 1998; Saviane et al., 1999). According to the ClC crystal structure, helix G has no direct contacts with the other subunit, as it lies behind the H and I helices (Dutzler et al., 2002). Nevertheless, a number of mutations in this helix are known to have significant effects on the common gate. For example, mutation C277S removes most of the common gating process of ClC-1 (Accardi et al., 2001; present work). Surprisingly, mutation of the adjacent cysteine residue to serine, C278S, didn't affect the common gate, but significantly reduced fast gating, increasing minimum P_o of the fast gate from ~0.05 to ~0.4. Other mutations in the G and H helices, T268M and I290M (Saviane et al., 1999), shifted P_o of both gates to more depolarizing potentials to a similar extent. In the P helix one of the mutants investigated, S541A, also shifted the fast gating process, in addition to its affect on the common gate, causing a shift in the $V_{1/2}$ of fast gating to more positive potentials. No known mutations in the I helix, or in the Q helix, affect fast gating.

Whole-cell current relaxations of the ClC-1 channel recorded during voltage steps to more negative potentials have been shown by direct single channel measurements to represent closure of the fast and common gates (Saviane et al., 1999). In the most comprehensive investigation to date of the kinetics of ClC-1 over a wide range of voltages Accardi and Pusch (2000) showed that at negative potentials the time constants of the fast and common gating relaxations are virtually voltage independent, whereas at more positive potentials the time constants become exponentially smaller, to the point where activation of the fast gate is too fast to be extracted from the activating currents. In their study a single exponential was able to describe activation of the common gate. In the present work, however, two exponential components were required to fit activation of the WT channel, and most mutant channels, recorded during voltage steps to more positive potentials. The time constant of the first component corresponded well with the time constant of the common gate activation reported previously by Accardi and Pusch (2000). The nature of the second component became apparent in the C277S mutant; while removing most of the slow exponential component from the current relaxation at negative potentials, this mutation also removed most of both exponential components at positive potentials.

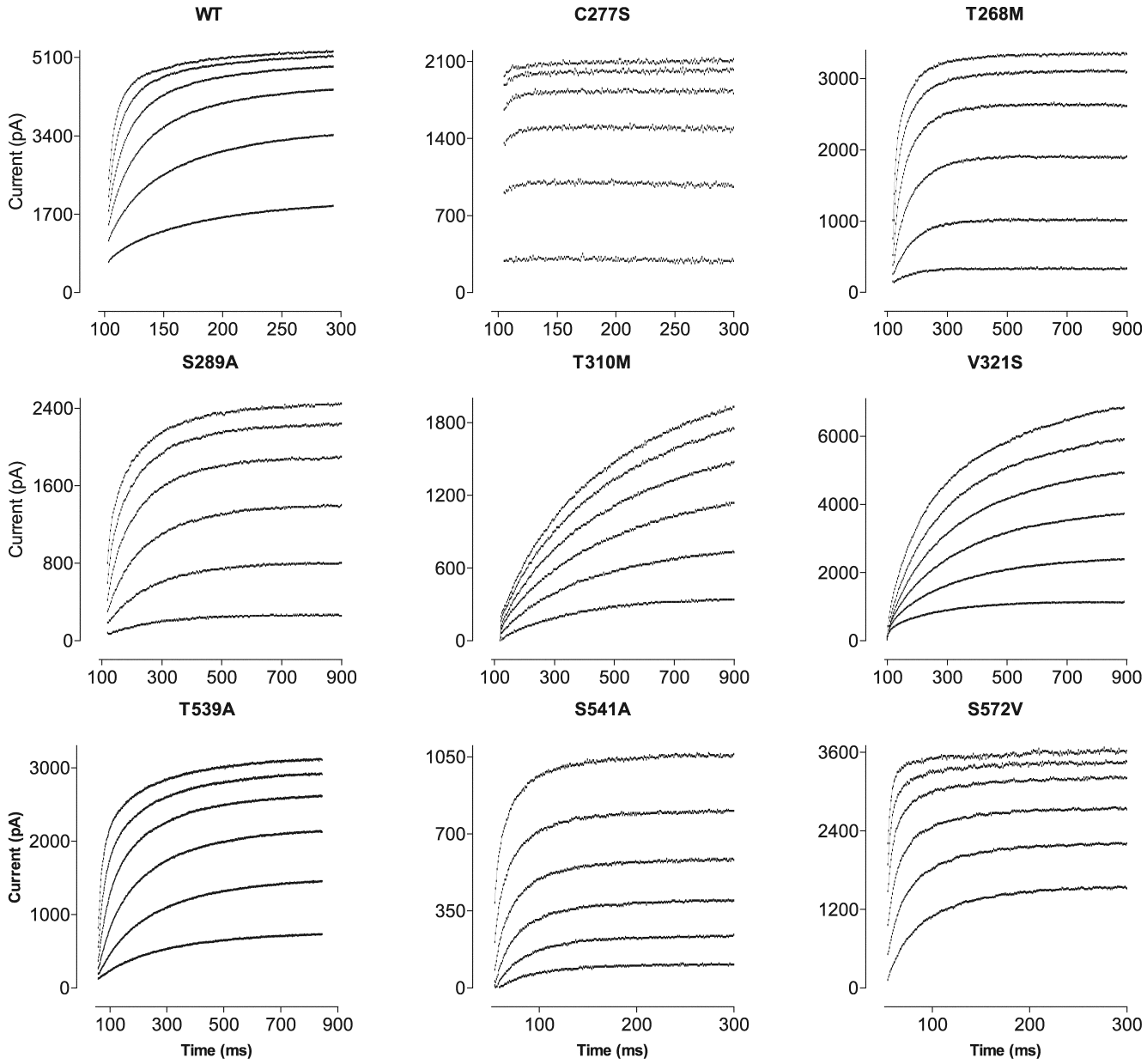


FIGURE 7. Kinetics of activation of WT and mutant ClC-1 channels. Currents were recorded in response to voltage steps ranging from 20 to 120 mV, in 20-mV increments, after a prepulse to −120 mV (not depicted).

Hence, it is likely that both of these two exponential components correspond to activation of the ClC-1 common gate, suggesting that at least a three-state kinetic model is required to describe the ClC-1 common gating process.

Open probabilities of both the fast and common gates of WT ClC-1 are voltage dependent, however at negative potentials they saturate at nonzero minimum values (Table I; Accardi and Pusch, 2000; Aromataris et al., 2001). Voltage dependence of the P_o of any channel arises from the voltage dependencies of its opening (α) and closing rates (β):

$$P_o(V) = \alpha(V)/[\alpha(V) + \beta(V)]. \quad (4)$$

Voltage dependence of α and β is described by the following equations:

$$\alpha(V) = \alpha(0)\exp(z_\alpha FV/RT) \quad (5a)$$

$$\beta(V) = \beta(0)\exp(z_\beta FV/RT), \quad (5b)$$

where $\alpha(0)$ and $\beta(0)$ are rates in the absence of voltage, and z is the gating charge of the corresponding gating transition. According to these equations a

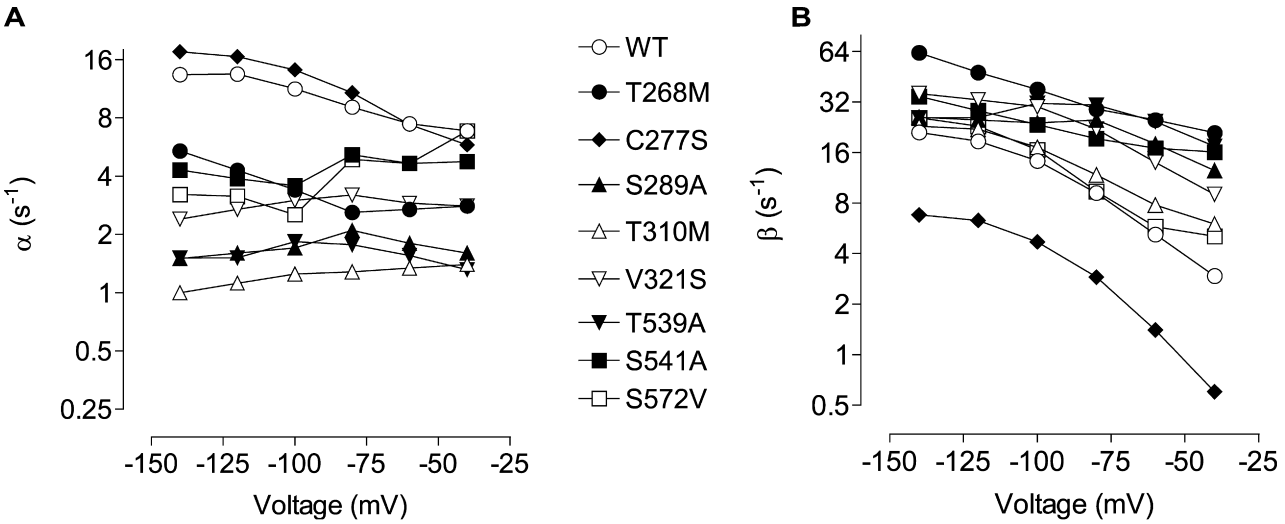


FIGURE 8. Rate constants for WT and mutant ClC-1 channel common gating at negative potentials. Opening (A) and closing (B) rate constants were calculated from open probabilities shown in Fig. 5 and time constants shown in Fig. 3, as described in MATERIALS AND METHODS (n = 3–10).

minimum P_o greater than zero is only possible if z_α and z_β become equal at some point, so that both rates vary with voltage in parallel. Calculations indicate that the opening and closing rates of the common gate of WT and mutant channels have the same voltage dependence at potentials negative to −80 mV, while at positive potentials the opening and closing rates have opposite voltage dependence. Such a pattern explains not only the nonzero minimum P_o, but also why the time constants of both fast and common gates of ClC-1 are virtually voltage independent at negative potentials, and become exponentially smaller at positive potentials (Accardi and Pusch, 2000). This case is similar to that seen for the fast gate of ClC-0 (Chen and Miller, 1996; Pusch et al., 2001).

To explain voltage dependence of the opening rate of the ClC-0 fast gate Chen and Miller (1996) proposed

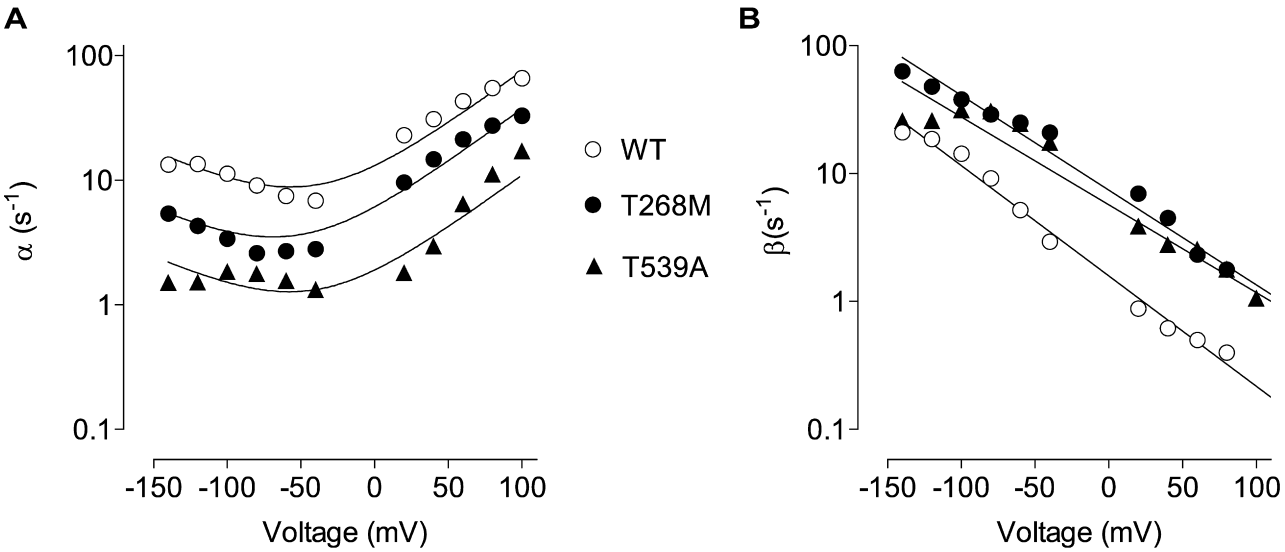
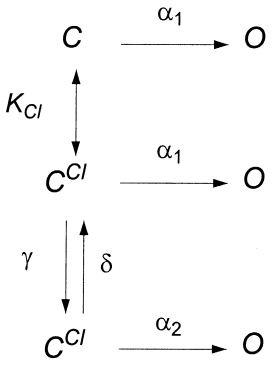


FIGURE 9. Voltage dependence of opening and closing rate constants of the common gating process for WT, T268M, and T539A ClC-1 channel. Data points for opening (A) and closing (B) rate constants at negative potentials are as in Fig. 8; data points at positive potentials are obtained as described in DISCUSSION. Opening rates are fitted with a two-exponential distribution (Eq. 8 combined with Eqs. 6 and 7) with the gating charges set to −0.3 and 0.5 for $\alpha_1(V)$ and $\gamma(V)$, respectively. The parameters of the fit were: $\alpha_1(0)$ (2.8, 0.97, and 0.41) and $\gamma(0)$ (10.3, 5.1, and 1.5) for WT, T268M, T539A, and T310M, respectively. Closing rates were fitted with a single-exponential distribution (Eq. 5 b).

the following model, with one unliganded (C) and two Cl^--liganded (C^{Cl}) closed states:



SCHEME I

Most of the channel's voltage dependence is postulated to come from the transitions between the two Cl^--liganded closed states; with $\alpha_2 >> \gamma$, δ, and α_1 at all accessible voltages. At negative potentials, the rate-limiting step is the hyperpolarization-favored opening process, with an opening rate α_1:

$$\alpha_1(V) = \alpha_1(0)\exp(z_{\alpha 1}FV/RT), \quad (6)$$

while at positive potentials Cl^- movement is the rate-limiting step, and the opening rate is determined by γ:

$$\gamma(V) = \gamma(0)\exp(z_{\gamma}FV/RT). \quad (7)$$

The observed overall opening rate is therefore the sum of α_1, and γ weighted by a factor determined by $[Cl^-]_o$, and the Cl^- dissociation constant, K_{Cl}:

$$\alpha(V) = \alpha_1(V) + \gamma(V)([Cl^-]_o/K_{Cl})/(1 + [Cl^-]_0/K_{Cl}). \quad (8)$$

This model describes well the experimental data on voltage and Cl^- dependence of ClC-0, and predicts that at high positive potentials activation of the fast gate of ClC-0 should follow a single exponential time course.

Voltage and Cl^- dependence of both the fast and the common gates of ClC-1 are similar to that of the fast gate of ClC-0 (Chen and Miller, 1996; Accardi and Pusch, 2000; Aromataris et al., 2001). In ClC-1, however, current activation between 20 and 120 mV follows a double exponential time course, suggesting a more complex mechanism for the common gating process of ClC-1 than that shown in Scheme I: either α_2 is not so much bigger than the other rate constants, or there is another closed state of the common gate. In the absence of single channel recordings, however, which would allow comprehensive modeling of the ClC-1 common gating process, the model suggested by Chen and Miller (Scheme I) can still be used for qualitative understanding of the changes in the common gating process caused by the mutations investigated in the present study.

For simplicity we therefore fitted ClC-1 activating currents with a single exponential, and used the corresponding time constants, together with the slow time constants of the deactivating currents, to calculate opening and closing rates for voltages between −140 and 100 mV. The results were fitted with Eqs. 5 b and 8, assuming that $([Cl^-]_o/K_{Cl})/(1 + [Cl^-]_o/K_{Cl})$ is a voltage-independent constant at a constant $[Cl^-]_o$, and can therefore be incorporated into $\gamma(0)$. The apparent gating charge $z_{\alpha 1}$ was set to −0.3 and z_{γ} to 0.5, which is comparable with that seen for the fast gate of ClC-0 (Chen and Miller, 1996), and is consistent with the results of Accardi and Pusch (2000). It is clear that mutations T268M and T539A shifted both opening rates of the common gate, α_1 and γ, along the Y-axis (Fig. 9). The observed shift of these opening rates could be attributed entirely to changes in the preexponential factors $\alpha_1(0)$ and $\gamma(0)$. In general, these factors depend on temperature and on the activation energy of the corresponding transitions in the absence of an electric field. In our case $\gamma(0)$ was also dependent on $[Cl^-]_o$ and K_{Cl}. It is unlikely, however, that K_{Cl} was altered, as not only $\gamma(0)$, but also $\alpha_1(0)$ was changed simultaneously by these mutations. Therefore it is probable that, compared with WT ClC-1, the energy of the unliganded closed state (Scheme I) is lower in these mutants, such that the activation energies of the opening transitions at either negative or positive potentials are increased. Consequently, channel closing becomes energetically more favorable. A decrease in the opening rate explains both the shift of $V_{1/2}$ to more depolarized potentials, and the decreased minimum P_o of the common gate of these mutant channels, as well as the slower channel activation kinetics seen at positive potentials. Mutations T268M and T310M shifted $V_{1/2}$ of the common gate to approximately the same extent; however, the kinetics of their activation at positive potentials are significantly different. This difference is explained by the fact that mutation T268M not only decreases the common gate opening rate, as T310M does, but also increases the closing rate. Although the increase in closing rate brings the same changes in the P_o as decrease of the opening rate, it makes the kinetics faster, not slower (Eq. 3 b).

An example of a mutation that showed only changes in the closing rate of the common gate is C277S. This mutation significantly decreased the closing rate of the common gate, with minimal effect on the opening rate which suggests that the energy of the closed state of this mutant is not different from that of the WT, but that the energy of the open state is lowered, such that the activation energy of the backward transition is greatly increased, and hence channel closing becomes energetically less favorable. An explanation for the increase of the minimum P_o of the common gate in C277S is thus provided.

In conclusion, this study has shown that the majority of mutations investigated at the interface between two monomers of ClC-1 are able to alter P_o and the kinetics of the common gate compared with WT channels. All of the effects on common gating caused by these mutations can be explained in terms of changes in either the common gate opening rate, the closing rate, or both, resulting from changes in the activation energies of the opening or closing transitions of the ClC-1 common gate. These results strongly support the hypothesis that the helices at the ClC-1 dimer interface are involved in the common gating process of ClC-1, however, it is likely the common gating process is not confined exclusively to the dimer interface and other regions of the channel may also be involved. While there is a clustering of naturally occurring dominant myotonic mutations in G, H, and I helicies, there are also some mutations in other regions that cause the condition, apparently through their effects on the common gate. These include: G200R (DE linker), A218T (Helix E), G230E (EF linker), P480L (MN linker), R894X, and P932L (COOH terminus) (see Pusch, 2002). Mutations that cause hyperpolarization-induced activation of the ClC-1 channel: S132C, D136G (Helix A), K231C (Helix F), and G499R (Helix N), also, presumably, either remove or drastically alter the ClC-1 common gating process, which suggests that mutations outside of the dimer interface may also affect ClC-1 common gating (Fahlke et al., 1995, 1997; Zhang et al., 2000; Wu et al., 2002). The precise mechanism of this complex and interesting phenomenon involving interaction of helicies at the interface with the other segments of the molecule is yet to be established.

We are grateful to Professor T.J. Jentsch of the Center for Molecular Neurobiology (Hamburg, Germany) for providing the human ClC-1 clone. The authors also wish to thank the Neurobiology laboratory at the Garvan Institute (Sydney, Australia), in particular Dr. Trevor Lewis and Dr. Kerrie Pierce, for their assistance with molecular biology techniques, and Lynette Cufone and Amie-Jane Palmer for technical assistance.

This work was supported by the Australian Research Council.

Olaf Andersen served as editor.

Submitted: 30 October 2002
Revised: 13 January 2003
Accepted: 13 January 2003

REFERENCES

Accardi, A., L. Ferrera, and M. Pusch. 2001. Drastic reduction of the slow gate of human muscle chloride channel (ClC-1) by mutation C277S. *J. Physiol.* 534:745–752.

Accardi, A., and M. Pusch. 2000. Fast and slow gating relaxations in the muscle chloride channel CLC-1. *J. Gen. Physiol.* 116:433–444.

Aromataris, E.C., G.Y. Rychkov, B. Bennetts, B.P. Hughes, A.H. Bretag, and M.L. Roberts. 2001. Fast and slow gating of ClC-1: differential effects of 2-(4-chlorophenoxy) propionic acid and dominant negative mutations. *Mol. Pharmacol.* 60:200–208.

Barry, P.H. 1994. JPCalc, a software package for calculating liquid junction potential corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for correction junction potential measurements. *J. Neurosci. Methods.* 51:107–116.

Bennetts, B., M.L. Roberts, A.H. Bretag, and G.Y. Rychkov. 2001. Temperature dependence of human muscle ClC-1 chloride channel. *J. Physiol.* 535:83–93.

Bretag, A.H. 1987. Muscle chloride channels. *Physiol. Rev.* 67:618–724.

Brugnoni, R., S. Galantini, P. Confalonieri, M.R. Balestrini, F. Cornelio, and R. Mantegazza. 1999. Identification of three novel mutations in the major human skeletal muscle chloride channel gene (CLCN1), causing myotonia congenita. *Hum. Mutat.* 14: 447.

Chen, T.Y., and C. Miller. 1996. Nonequilibrium gating and voltage dependence of the ClC-0 Cl^- channel. *J. Gen. Physiol.* 108:237–250.

Dutzler, R., E.B. Campbell, M. Cadene, B.T. Chait, and R. MacKinnon. 2002. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature.* 415:287–294.

Fahlke, C., R. Rudel, N. Mitrovic, M. Zhou, and A.L. George. 1995. An aspartic acid residue important for voltage-dependent gating of human muscle chloride channels. *Neuron.* 15:463–472.

Fahlke, C., H.T. Yu, C.L. Beck, T.H. Rhodes, A.L. George. 1997. Pore-forming segments in voltage-gated chloride channels. *Nature.* 390:529-532.

Fong, P., A. Rehfeldt, and T.J. Jentsch. 1998. Determinants of slow gating in ClC-0, the voltage-gated chloride channel of *Torpedo marmorata*. *Am. J. Physiol.* 274:C966–C973.

Furman, R.E., and R.L. Barchi. 1978. The pathophysiology of myotonia produced by aromatic carboxylic acids. *Ann. Neurol.* 4:357–365.

Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 77:51–59.

Jentsch, T.J., V. Stein, F. Weinreich, and A.A. Zdebik. 2002. Molecular structure and physiological function of chloride channels. *Physiol. Rev.* 82:503–508.

Kubisch, C., T. Schmidt-Rose, B. Fontaine, A.H. Bretag, and T.J. Jentsch. 1998. ClC-1 chloride channel mutations in myotonia congenita: variable penetrance of mutations shifting the voltage dependence. *Hum. Mol. Genet.* 7:1753–1760.

Kwiecinski, H., F. Lehmann-Horn, and R. Rudel. 1988. Drug-induced myotonia in human intercostal muscle. *Muscle Nerve.* 11: 576–581.

Lin, Y.W., C.W. Lin, and T.Y. Chen. 1999. Elimination of the slow gating of ClC-0 chloride channel by a point mutation. *J. Gen. Physiol.* 114:1–12.

Ludewig, U., M. Pusch, and T.J. Jentsch. 1996. Two physically distinct pores in the dimeric ClC-0 chloride channel. *Nature.* 383: 340–343.

Middleton, R.E., D.J. Pheasant, and C. Miller. 1994. Purification, reconstitution, and subunit composition of a voltage-gated chloride channel from *Torpedo* electroplax. *Biochemistry.* 33:13189–13198.

Middleton, R.E., D.J. Pheasant, and C. Miller. 1996. Homodimeric architecture of a ClC-type chloride ion channel. *Nature.* 383:337–340.

Pusch, M. 2002. Myotonia caused by mutations in the muscle chloride channel gene CLCN1. *Hum. Mutat.* 19:423–434.

Pusch, M., A. Accardi, A. Liantonio, L. Ferrera, A. De Luca, D.C. Camerino, and F. Conti. 2001. Mechanism of block of single protopores of the *Torpedo* chloride channel ClC-0 by 2-(*p*-chlorophenoxy)butyric acid (CPB). *J. Gen. Physiol.* 118:45–62.

Pusch, M., U. Ludewig, and T.J. Jentsch. 1997. Temperature depen-

dence of fast and slow gating relaxations of ClC-0 chloride channels. *J. Gen. Physiol.* 109:105–116.

Pusch, M., U. Ludewig, A. Rehfeldt, and T.J. Jentsch. 1995a. Gating of the voltage-dependent chloride channel CIC-0 by the permeant anion. *Nature.* 373:527–531.

Pusch, M., K. Steinmeyer, and T.J. Jentsch. 1995b. Mutations in dominant human myotonia congenita drastically alter the voltage dependence of the CLC-1 chloride channel. *Neuron.* 15: 1455–1463.

Rychkov, G.Y., M. Pusch, D. St. Astill, M.L. Roberts, T.J. Jentsch, and A.H. Bretag. 1996. Concentration and pH dependence of skeletal muscle chloride channel ClC-1. *J. Physiol.* 497:423–435.

Rychkov, G.Y., M. Pusch, M.L. Roberts, T.J. Jentsch, and A.H. Bretag. 1998. Permeation and block of the skeletal muscle chloride channel, ClC-1, by foreign anions. *J. Gen. Physiol.* 111:653–665.

Saviane, C., F. Conti, and M. Pusch. 1999. The muscle chloride channel ClC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *J. Gen. Physiol.* 113:457–468.

Steinmeyer, K., C. Lorenz, M. Pusch, M.C. Koch, and T.J. Jentsch. 1994. Multimeric structure of ClC-1 chloride channel revealed by mutations in dominant myotonia congenita (Thomsen). *EMBO J.* 13:737–743.

Weinreich, F., and T.J. Jentsch. 2001. Pores formed by single subunits in mixed dimers of different CLC chloride channels. *J. Biol. Chem.* 276:2347–2353.

Wollnik, B., C. Kubisch, K. Steinmeyer, and M. Pusch. 1997. Identification of functionally important regions of the muscular chloride channel ClC-1 by analysis of recessive and dominant myotonic mutations. *Hum. Mol. Genet.* 6:805–811.

Wu, F.F., A. Ryan, J. Devaney, M. Warnstedt, Z. Korade-Mimics, B. Poser, M.J. Escriva, E. Pegoraro, A.S. Yee, K.J. Felice, et al. 2002. Novel CLCN1 mutations with unique clinical and electrophysiological consequences. *Brain.* 125:2392–2407.

Zhang, J., M.C. Sanguinetti, H. Kwiecinski, and L.J. Ptacek. 2000. Mechanism of inverted activation of ClC-1 channels caused by novel myotonia congenita mutation. *J. Biol. Chem.* 275:2999–3005.