ClC-1 chloride channel mutations in myotonia congenita: variable penetrance of mutations shifting the voltage dependence

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Mutations in the ClC-1 muscle chloride channel cause either recessive or dominant myotonia congenita. Using a systematic screening procedure, we have now identified four novel missense mutations in dominant (V286A, F307S) and recessive myotonia (V236L, G285E), and have analysed the effect of these and other recently described mutations (A313T, I556N) on channel properties in the Xenopus oocyte expression system. Mutations V286A, F307S and A313T displayed a ‘classical’ dominant phenotype: their voltage dependence was shifted towards positive potentials and displayed a dominant-negative effect by significantly imparting a voltage shift on mutant–wild-type heteromeric channels as found in heterozygous patients. In contrast, the recessive mutation V236L also shifted the voltage dependence to positive values, but co-expression with wild-type ClC-1 gave almost wild-type currents. I556N, a mutation found in patients with benign dominant myotonia, drastically shifts the voltage dependence, but only a slight shift is seen when co-expressed with wild-type ClC-1. Thus, the voltage dependence of mutant heteromeric channels is not always intermediate between those of the constituent homomeric channel subunits, a conclusion further supported by mixing different ClC-1 mutants. These complex interactions correlate clinically with various inheritance patterns, ranging from autosomal dominant with various degrees of penetrance to autosomal recessive.

INTRODUCTION

Non-dystrophic myotonia congenita is an inherited disorder of the skeletal muscle characterized by a delayed muscle relaxation after contraction (for a recent review, see ref. 1). Myotonia congenita may be inherited in an autosomal dominant (Thomsen disease, OMIM 160800) or recessive (Becker disease, OMIM 255700) manner. As shown first for a mouse model of the disease (2), both the recessive and the dominant forms of human myotonia are due to mutations in the gene (CLCN1) encoding the major skeletal muscle chloride channel ClC-1 (3,4). ClC-1 is important for the normal repolarization of muscle action potentials. Its loss of function renders the plasma membrane hyperexcitable and leads to the typical ‘myotonic runs’ seen in electromyograms of myotonic patients. More than 30 mutations have now been described in human myotonia. They are scattered over nearly the entire length of the channel protein. Total loss-of-function mutations, like those truncating the protein before the cytoplasmic C-terminus, always lead to recessive disease, as do many missense mutations, some of which reduce the single channel conductance. Mutations found in dominant myotonia congenita exert dominant-negative effects on the co-expressed wild-type subunits (5). This requires that ClC-1 is a (homo)multimer. Indeed, the homologous Torpedo channel ClC-0 (6) is a homodimer with one pore per subunit (7,8). There are convincing data (9) that ClC-1 is a homodimer as well, although the strong dominant-negative effect of some mutations suggested even higher aggregates (5). Most dominant mutations analysed so far (10,11) shifted the voltage dependence of the ClC-1 chloride channel to positive potentials, where it can no longer contribute to the repolarization of action potentials. When mutant and wild-type channel subunits were co-expressed at a 1:1 ratio to mimic the situation in heterozygous patients, the half-activation of the resulting heteromeric channels by voltage occurred roughly halfway between the wild-type and pure mutant channels. This shift was still large enough to prevent ClC-1 from significantly repolarizing the action potential, fully explaining the dominant-negative effect underlying dominant myotonia. However, some mutations do not conform easily to the simple dominant/recessive scheme. Thus, some mutations seem to have a reduced penetrance, and other mutations were found in both dominant and recessive pedigrees (5,12–14). While this may

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RESULTS

Identification of novel CLCN1 mutations

To optimize screening procedures for CLCN1 mutations, we (i) developed a hierarchy of screening exons, based on the likelihood of harbouring mutations; (ii) amplified certain short exons in pairs together with intervening short introns; and (iii) directly sequenced both strands of PCR products, which reliably detects even heterozygous mutations in DNA stretches as long as 700 bp.

Analysis of CLCN1 mutations identified so far (Table 1) shows that at least two different mutations have been described in exons 3, 4, 5, 8, 9, 11, 12, 13 and 15, whereas no mutations have been found so far in exons 6, 10, 16 and 18–22. We therefore screened the complete coding region and splice sites by examining exons in the following order: exons 8, 9, 13/14, 4/5, 11/12, 15, 23, 3, 7, 2, 1, 6, 10, 16, 17, 18, 19/20, 21/22.

Sequencing both strands of the PCR products revealed mutations in all six patients. Both patients with bona fide dominant myotonia were heterozygous for novel mutations in exon 8: in patient no. 6245, a T→C transition in the triplet encoding Val286 changed it to alanine (V286A), while in patient no. 6508 a T→C transition changed Phe307 to serine (F307S).

Both residues are highly conserved in the CLC family (Fig. 1B). Neither of these mutants conformed to previous, ‘classical’ behaviours of recessive and dominant mutations. In contrast, a recessive mutation (V236L) as well as a dominant mutation with reduced penetrance (I556N) shift the voltage dependence of ClC-1 to positive potentials, but do not markedly impart this property on heteromeric mutant/wild-type channels. Further, co-expression of several different mutants shows that the shift of voltage dependence often differs markedly from being halfway between those of the corresponding homomeric channels. This sheds an interesting light on ClC-1 subunit interactions, and helps to explain the differences in inheritance patterns and penetrance.

Table 1. Location of CLCN1 mutations leading to myotonia congenita

<table>
<thead>
<tr>
<th>Exon</th>
<th>Domain</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nt</td>
<td>3a→t (r)5 (donor splice site intron 1)</td>
</tr>
<tr>
<td>2</td>
<td>Nt</td>
<td>Q68X (r)44</td>
</tr>
<tr>
<td>3</td>
<td>Nt, D1</td>
<td>R105C (r)12</td>
</tr>
<tr>
<td>4</td>
<td>D2</td>
<td>Y150C (r)11,35</td>
</tr>
<tr>
<td>5</td>
<td>D2/D3</td>
<td>G200R (d)11,35</td>
</tr>
<tr>
<td>6</td>
<td>D4</td>
<td>Y261C (r)13,14,22,34</td>
</tr>
<tr>
<td>7</td>
<td>D5</td>
<td>Y261C (r)11,35</td>
</tr>
<tr>
<td>8</td>
<td>D5/D6</td>
<td>G285E (r)present study</td>
</tr>
<tr>
<td>9</td>
<td>D7</td>
<td>G399a (r)29 (donor splice site intron 8)</td>
</tr>
<tr>
<td>11</td>
<td>D8</td>
<td>E291K (r)10,12</td>
</tr>
<tr>
<td>12</td>
<td>D9</td>
<td>R262insC (r)12</td>
</tr>
<tr>
<td>13</td>
<td>D9/D10</td>
<td>del(4 bp)1278–1285 (r)36</td>
</tr>
<tr>
<td>14</td>
<td>D10/D11</td>
<td>G240R (r)12</td>
</tr>
<tr>
<td>15</td>
<td>D11/D12</td>
<td>M485V (r)11,12,present study</td>
</tr>
<tr>
<td>17</td>
<td>Ct</td>
<td>E291K (r)10,12</td>
</tr>
<tr>
<td>23</td>
<td>Ct</td>
<td>R894X (d)12,14,22,34</td>
</tr>
</tbody>
</table>

The genomic region encompassing exons 8–12, we tested whether both mutations were on the same chromosomes. The wild-type PCR product (∼2600 bp) is cut by SfiI, but this site is destroyed by the G285E mutation. Sequencing of the SfiI-resistant product revealed the A437T mutation, showing that both mutations are located on the same chromosome and are at linkage disequilibrium. This also further strengthens the notion that A437T is a neutral polymorphism. Screening of the remaining CLCN1 exons, however, did not reveal any other mutation.

*CLCN1 exon number (29).

*Part of the protein encoded by these exons. Nt, N-terminus; Ct, cytoplasmic C-terminus; D1–D13, CLC chloride channel domains (mostly transmembrane domains) according to refs 6 and 33.

*Described mutations in CLCN1; in parentheses: d, dominant; r, recessive; d/r includes mutations found in both dominant and recessive pedigrees and, probably overlapping, dominant mutations with reduced penetrance; superscripts refer to work in which these mutations were described or analysed.
Figure 1. (A) Schematic diagram showing the location of dominant (or partially dominant) human mutations in the ClC-1 chloride channel (for phenotypes, see Table 1). The topology is according to Schmidt-Rose and Jentsch (33). Except for G230E and R894X, all these mutations shift the voltage dependence to the right. In addition, Q552R significantly slows the activation by depolarization (10). (B) Sequence conservation in regions where mutations described in this work are located; representatives of the three different branches of mammalian CLC channels are: hClC-1, the skeletal muscle chloride channel analysed here; hClC-5, the kidney chloride channel whose mutations cause Dent’s disease; hClC-7, a ubiquitously expressed putative chloride channel. In addition, the homologous sequences from the yeast scClC (Gef1) and a protein from Escherichia coli, ecClC, are shown.

In patient no. 6237, we identified an intronic splice site mutation at the boundary of exon 1 to intron 1 (an A→T transversion at position –3). An identical mutation was described previously in four unrelated myotonic families, two of which were homozygous for this mutation (16). Except for a known, silent polymorphism at Thr87, no second mutation could be identified in this patient.

Functional analysis of myotonic mutations

To gain insights into the mechanisms by which these mutations cause recessive or dominant myotonia, we studied the electrophysiological properties of the mutated channels in the Xenopus oocyte expression system. We also included two clinically interesting mutations (A313T and I556N) recently described by Plassart-Schiess et al. (14). The myotonic phenotype associated with these mutations appears variable, as the A313T mutation was found in one recessive and another dominant pedigree, while the I556N mutation caused a rather benign phenotype compatible with a dominant mutation having a reduced penetrance.

We first analysed the mutations which cause fully dominant myotonia (V286A, F307S) and those leading to partially dominant myotonia (A313T, I556N). When expressed in Xenopus oocytes, all these mutants generated chloride currents which were activated by depolarization (see Fig. 2 for F307S). Their amplitudes were in the range of wild-type ClC-1 currents. However, while wild-type ClC-1 is half-maximally open at $V_{1/2} = –20 \pm 2$ (SEM) mV, the open probability of these mutants is shifted by >50 mV towards positive voltages. Half-maximal
open probabilities occurred at $V_{1/2} = 41 \pm 4$ mV for V286A, at $V_{1/2} = 32 \pm 6$ mV for F307S, at $V_{1/2} = 39 \pm 6$ mV for A313T and at $V_{1/2} = 31 \pm 3$ mV for the I556N mutant. Similar shifts in voltage dependence have been found previously with other ClC-1 mutations causing dominant myotonia (10,11).

The voltage dependence of these mutant channels is shifted to such a degree that they can no longer contribute to the repolarization of action potentials. In order to cause dominant myotonia, however, they have to exert a dominant-negative effect on wild-type ClC-1 subunits encoded by the normal allele in heterozygous patients. We therefore co-expressed these mutants with wild-type ClC-1 in oocytes and examined the voltage dependence of resulting currents (Figs 2C and F, and 3). The V286A, F307S and A313T mutants led to a large shift in voltage dependence also on a 1:1 co-expression with wild-type ClC-1 (Fig. 3, Table 2). As found previously with other dominant mutations (e.g. I290M) (10), $V_{1/2}$ was roughly halfway between those of pure mutant or wild-type channels, or was even closer to that of the mutant (in particular for F307S). This fully explains their dominant mode of inheritance. In contrast, when I556N was co-expressed with wild-type ClC-1, the voltage dependence of the resulting current was rather close to that of the wild-type channel, predicting only a slight dominant-negative effect.

We next investigated the recessive mutations V236L and G285E. With the latter mutation, we were unable to detect expression of chloride currents. Co-expression with wild-type ClC-1 did not change these currents qualitatively and there was no detectable dominant-negative effect on current amplitudes, which is fully compatible with recessive inheritance. Interestingly, and in that respect resembling the dominant mutations, V236L shifted the voltage dependence of ClC-1 to positive potentials. However, this shift was less pronounced ($V_{1/2} = 9 \pm 4$ mV), and macroscopic conductance was significantly reduced (by a factor of 3) when compared with wild-type. When co-expressed with wild-type ClC-1, current amplitudes were $\sim 50\%$ of wild-type, and had wild-type voltage dependence. This is again compatible with a recessive mutation.

Thus, mutations shifting the voltage dependence of ClC-1 activation can behave both recessively and dominantly, depending on the quantity of the shift and on the extent to which they can impart their voltage dependence on wild-type subunits. In addition, as for V236L, a reduction in current amplitudes may come into play. Our data show that the voltage dependence of mutant–wild-type heteromeric channels is not always intermediate between that of its components, but depends critically on which subunits are mixed. To investigate this further, we co-injected in pairs several mutants leading to different shifts in voltage dependence. Surprisingly, co-expression of I556N with V236L led to a larger shift of $V_{1/2}$ (to roughly $+40$ mV) than observed with any of its components (Fig. 3). Current amplitudes were close to wild-type (Table 2).

Figure 2. Electrophysiological analysis of mutations shifting the voltage dependence of ClC-1 and their effect on wild-type ClC-1 in co-expression experiments designed to mimic the situation in heterozygous patients. Channels were expressed in Xenopus oocytes and examined by two-electrode voltage clamping. (A) Pulse protocol. Starting from a holding potential of $-100$ mV, oocytes were clamped for 600 ms to values between $+100$ and $-140$ mV in steps of 20 mV. (B) Current traces from a typical oocyte expressing wild-type ClC-1. (C) A 1:1 co-injection of wild-type and F307S ClC-1 and (D) pure mutant F307S ClC-1. As shown in (E), which is a plot of open probability (determined by tail currents) as a function of voltage, the mutation shifts the voltage dependence by $\sim 50$ mV to positive values, and a large shift is also seen on 1:1 co-expression (triangles), fully explaining the dominant character of this mutation. (F) Co-expression of wild-type ClC-1 and the I556N mutation as in heterozygous patients and (G) currents from the pure mutant I556N channel. As seen in (H) I556N also dramatically shifts the voltage dependence to positive potentials, but, in contrast to the F307S mutation, this shift on co-expression with wild-type is only small, explaining the decreased penetrance of this mutation in patients. Boltzman curves in (E) and (H) were calculated using equation (1) and mean values of $z, V_{1/2}$ and $I_{max}$ were obtained from experiments with $n \geq 10$ oocytes. Experimental values for $I_0$ were $\sim 10\%$ of $I_{max}$ varying with the batch of oocytes (data not shown). No correlation of $I_0$ with the introduced mutations was observed. We assume that for hyperpolarizing voltages approaching $\sim -\infty$, ClC-1 currents are 0, the remaining currents being due only to endogenous oocyte channels and leakage. Therefore, $I_0$ was subtracted in the fits shown.
Parameters of currents expressed from wild-type ClC-1 and the mutants investigated in this work.

<table>
<thead>
<tr>
<th>Voltage (mV) at $P_{\text{open}} = 0.5$</th>
<th>Apparent gating charge $z$</th>
<th>Conductance (µS) at $P_{\text{open}} = 0.5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (WT)</td>
<td>$-20 \pm 2$</td>
<td>$0.94 \pm 0.03$</td>
</tr>
<tr>
<td>V236L</td>
<td>$+9 \pm 4$</td>
<td>$0.97 \pm 0.06$</td>
</tr>
<tr>
<td>V236L + WT</td>
<td>$-19 \pm 3$</td>
<td>$0.92 \pm 0.02$</td>
</tr>
<tr>
<td>V236L + M485V</td>
<td>$-10 \pm 2$</td>
<td>$0.90 \pm 0.02$</td>
</tr>
<tr>
<td>G285E</td>
<td>no current</td>
<td>no current</td>
</tr>
<tr>
<td>G285E + WT</td>
<td>$-21 \pm 5$</td>
<td>$1.03 \pm 0.04$</td>
</tr>
<tr>
<td>V286A + WT</td>
<td>$+41 \pm 4$</td>
<td>$0.65 \pm 0.02$</td>
</tr>
<tr>
<td>V286A + WT</td>
<td>$+17 \pm 2$</td>
<td>$0.94 \pm 0.03$</td>
</tr>
<tr>
<td>F307S + WT</td>
<td>$+32 \pm 6$</td>
<td>$0.97 \pm 0.04$</td>
</tr>
<tr>
<td>F307S + WT</td>
<td>$+23 \pm 5$</td>
<td>$0.88 \pm 0.02$</td>
</tr>
<tr>
<td>A313T + WT</td>
<td>$+39 \pm 6$</td>
<td>$1.13 \pm 0.03$</td>
</tr>
<tr>
<td>A313T + V236L</td>
<td>$+17 \pm 3$</td>
<td>$0.79 \pm 0.05$</td>
</tr>
<tr>
<td>I556N</td>
<td>$+31 \pm 3$</td>
<td>$1.02 \pm 0.07$</td>
</tr>
<tr>
<td>I556N + WT</td>
<td>$-11 \pm 1$</td>
<td>$1.06 \pm 0.06$</td>
</tr>
<tr>
<td>I556N + V236L</td>
<td>$+40 \pm 1$</td>
<td>$0.86 \pm 0.02$</td>
</tr>
<tr>
<td>I556N + M485V</td>
<td>$-19 \pm 3$</td>
<td>$0.99 \pm 0.04$</td>
</tr>
</tbody>
</table>

Open probabilities of channels were measured by tail current analysis as described in Materials and Methods and fitted with a Boltzmann equation. Parameters obtained from these fits are given as $V_{1/2}$ (the voltage at which 50% of the channels are open, i.e. $P_{\text{open}} = 0.5$), and the apparent gating charge $z$ (see equation 1). The last column gives the macroscopic conductance at $V_{1/2}$, which is a measure of the ‘expressibility’ of the channel and is independent of the shift in voltage dependence observed in mutants. These values depend on several parameters, e.g. protein stability, transport to the surface and single channel conductance.

We also co-injected V236L with the M485V mutant because patient no. 1057 is compound heterozygous for these mutations. M485V was shown previously by noise analysis to reduce ClC-1 single-channel conductance from 1.5 to 0.3 pS (11), explaining the reduced macroscopic currents seen in the present experiments (Table 2). Moreover, M485V is strongly inwardly rectifying and deactivated incompletely at negative voltages. M485V had no dominant-negative effect on wild-type ClC-1 (11). When we co-expressed M485V with V236L, currents activated at voltages more positive than wild-type ClC-1 (Fig. 3). Current amplitudes were less than those with the wild-type + V236L co-injection (Table 2), explaining myotonia in this compound heterozygote.

**DISCUSSION**

Chloride channel myotonia may serve as a paradigm for genetic diseases in which different mutations in a single gene can lead to either recessive or dominant patterns of inheritance. This distinction, however, is not clear-cut in myotonia, as some mutations can be recessive in one pedigree and dominant in another. Probably all these mutations have a weak dominant-negative effect, leading to a reduced penetrance in patients. Ion channel diseases such as myotonia make valuable models because the effects of mutations can be studied in considerable detail *in vitro*.

Mutations in many regions of a protein can lead to a loss of function, and indeed ClC-1 mutations found in myotonia are scattered over much of the protein. Except for a short truncation after the conserved cytoplasmic domain D13 (R894X), which leads to a weakly dominant phenotype (12), all other truncations (occurring before the end of the transmembrane spans) lead to recessive myotonia. Missense mutations, however, may cause recessive or dominant myotonia. It is impossible to predict from the position of missense mutations whether they will be dominant or recessive; the dominant I290M mutation is adjacent to the recessive E291K (10), and here we have identified two new adjacent mutations (G285E and V286A), which are also recessive and dominant, respectively.

While it is true that myotonic mutations can be found in many regions of ClC-1, they are not evenly distributed over the channel. As expected for a channel protein, there is a clear predilection for the transmembrane block. Even within this block, some regions harbour more mutations than others. Interestingly, there seems to be a cluster of dominant mutations in the D5–D6 region (exon 8;
with mutations V286A, I290M, F307S, A313T and R317Q) (Fig. 1A). However, there are also dominant mutations in the D9–D12 block [the P480L mutation found in Thomsen’s own pedigree (5)], in Q552R (17) and the partially dominant I556N (14), and in the D2–D3 linker [G200R (11)].

Finding mutations in CIC-1 can be arduous as the channel is encoded by 23 exons. By amplifying two exons with short intervening introns together in one PCR, and by establishing a hierarchy of screening based on the likelihood of finding mutations in particular exons, we have established an efficient screening procedure. By screening just 12 PCR products (representing exons 8/9, 13/14, 4/5, 11/12, 15, 23, 3, 7, 2, 6, 1 and 17), all CIC-1 mutations described so far would be detected. On the other hand, and in line with previous studies (12), we did not find mutations of the second allele of some patients with presumed recessive myotonia. Possible reasons may include that the mutations identified are partially dominant with decreased penetrance (which may not be reflected in the oocyte system), that there are mutations in the promotor or in introns which have not been sequenced or that there may be microdeletions of the second allele.

A total loss of function of CIC-1 on one allele leads to recessive disease, as demonstrated by mutations leading to early truncations of the channel protein. Dominant mutations decrease chloride conductance below a critical threshold of ∼30% by a dominant-negative effect. This implies that mutant dominant CIC-1 subunits can still assemble efficiently with wild-type subunits, thereby compromising the function of the mutant–wild-type heteromer.

The dominant mutations investigated here (V286A, F307S and A313T) drastically shifted the voltage dependence of CIC-1 to positive potentials, and a similar, though less pronounced, shift was also seen with heteromeric mutant–wild-type channels. This explains the dominant-negative effect, because the altered voltage dependence of the resulting heteromers prevents these channels from repolarizing muscle action potentials efficiently. Such a shift in voltage dependence has been found previously with other dominant mutations (10,11) and is the most common mechanism by which mutations in Thomsen’s disease exert a dominant-negative effect.

Dominant mutations shifting the voltage dependence are found in several regions of IICIC-1. Further, a mutation that shifts the CIC-1 voltage dependence in the myotonic goat is close to the cytoplasmic C-terminus (a dominant-negative effect has not yet been shown) (18). Hence these mutations do not affect a putative voltage sensor, which may not even exist in this channel class whose gating depends on the permeant anion (19–21). These mutations may rather affect the energy difference between the closed and the open state of the channel, and this may be achieved by alterations in many parts of the protein.

G230E (22) and R894X (23) are the only known dominant mutations which do not shift the CIC-1 voltage dependence to positive voltages. However, both mutations have reduced penetrance and were found both in dominant and recessive pedigrees (12,13). Electrophysiological analysis in oocytes revealed that both mutations have only a weak dominant-negative effect (5,12). The G230E mutation leads to changes in channel kinetics and ion selectivity in hetero- and homooligomers (5,24), while the R894X mutation leads to reduced currents which otherwise have wild-type characteristics (12). It was speculated that the truncation after domain D13 (R894X) reduces protein stability or trafficking to the surface (12).

Recessive mutations require that mutated subunits either do not interact with their wild-type counterparts, or that the function of resulting mutant–wild-type heteromers is not compromised sufficiently to cause disease. While intramolecular associations of CIC-1 domains has been investigated (25), the domains mediating the association of CLC subunits are not yet known. Since truncations within the transmembrane block cause recessive disease, segments after D9 may be required for oligomerization. Our experiments do not allow us to decide whether the recessive mutant G285E, which did not generate currents in our experiments, is unable to associate with wild-type subunits, or whether potential heteromeric channels yield ∼50% of wild-type currents. It is interesting to note that another recessive mutation (D136G) probably hinders the intramolecular assembly of CIC-1 channels when expressed from artificially 'split' channels (25). Previous co-injection experiments using M485V and wild-type CIC-1 were also unable to determine whether this mutant, whose single-channel conductance and gating is changed, can assemble with wild-type subunits (11).

One of the most interesting aspects of the present work is that not all mutations causing a positive shift in voltage dependence are necessarily dominant. It is immediately apparent that a moderate shift (of the order of 10–20 mV) may show a recessive pattern of inheritance if the resulting heteromeric channels have an intermediate voltage dependence, as previously found for several mutants (10,11). The shift with the homomeric V236L channel (∼30 mV) may be just at the limit of causing myotonia by a dominant-negative mechanism. Interestingly, co-expression with wild-type CIC-1 did not reveal any shift in voltage dependence. However, given the reduced current amplitudes of the homomeric mutant V236L channel, it cannot be excluded that V236L channels do not associate with wild-type channels; in that case, macroscopic currents would be dominated by wild-type CIC-1.

The situation is more interesting with I556N. The shift in voltage dependence is drastic, and current amplitudes are comparable with that of wild-type CIC-1. Nonetheless, co-expression with wild-type yields only a minor shift in the activation voltage, strongly suggesting that the $V_{1/2}$ of the heteromeric channel is only slightly shifted. This also argues against the possibility that the resulting currents are due just to a linear superposition of separate wild-type and mutant channels, and also the slope of the voltage dependence (reflected in the apparent gating charge $\tilde{\epsilon}$; Table 2) argues for the formation of heteromeric mutant–wild-type channels (10).

Thus, $V_{1/2}$ of heteromers is not intermediate between those of the individual subunits. This conclusion is supported further by our co-expression of I556N and V236L; the resulting shift is larger than that of any of its components. Thus, voltage-depend-ent gating depends in a complicated manner on the individual subunits, and cannot be predicted from the properties of the homomeric subunits alone.

The *Torpedo* channel CIC-0 is a dimeric, ‘double-barrelled’ channel with two identical pores which are formed by one subunit each (7,8). Both pores of the channel have an individual gate, and analysis of mutant–wild-type heteromers has shown that the gating, single channel conductance, rectification and ion selectivity of an individual pore is largely independent of the second,
attached pore (7,8,26). However, there is a second, ‘slow’ gate which acts on both pores together. Certain mutations which affect this common gate also affect this gate in the mutant–wild-type heteromeric channel (8), allowing for a dominant effect on wild-type subunits. Unfortunately, single-channel analysis of CIC-1 is nearly excluded due to its low single-channel conductance of 1–1.5 pS (27). Thus, while it seems reasonable to assume that CIC-1 is a double-barrelled channel similar to CIC-0, this has not yet been shown directly. Nonetheless, it is tempting to speculate that the dominant effect on gating as exemplified by several of the myotonic mutations is by a mechanism similar to the common gate of CIC-0.

From a more clinical point of view, the classification of myotonia congenita into a dominant form (Thomsen’s disease) and a recessive form (Becker type) is quite artificial, and several mutations behave as partially dominant with a reduced penetrance. The oocyte expression system is well suited to explaining these different modes of inheritance, but there are some limitations. For instance, the present analysis would predict a fully dominant phenotype for the A313T mutation, which clinically was associated rather with reduced penetrance (14). This is reminiscent of the Q552R mutation, which displayed a strongly dominant phenotype in the oocyte system (10), but was associated with a clinically benign form of myotonia (17). Nonetheless, structure–function analysis in the Xenopus oocyte system and comparison with the clinical phenotype has now established myotonia congenita as a well-understood paradigm for a monogenic disease with differing patterns of inheritance.

MATERIALS AND METHODS

Patients

Five patients from Australia of Caucasian descent (patients nos 6193, 6237, 6245, 6267 and 6508) and one Caucasian patient from Germany (no. 1057) were screened for CLCN1 mutations. All patients investigated gave informed consent and were diagnosed according to common diagnostic criteria (28). Based on anamnestic data, two patients (nos 6245 and 6508) are likely to have dominant myotonia congenita, but no DNA samples were available from other members of these families.

Mutation analysis

Genomic DNA was extracted from whole blood. Intronic primers described previously (29) were used to amplify CLCN1 exons, and mutations were sought by directly sequencing the PCR products. To streamline the procedure, several small exons were amplified together if the intervening introns were small enough (exons 4/5, 8/9, 11/12, 13/14, 19/20 and 21/22). This resulted in PCR products of no more than 674 bp. Genomic DNA (100 ng) was amplified in a 50 µl volume containing 1 U of Taq DNA polymerase (Boehringer Mannheim), 15 pmol of each primer and 500 µM of each dNTP, using the buffer supplied by the manufacturer (1.5 mM MgCl2) and a 2400 Thermocycler (Perkin Elmer). Primer sequences and PCR conditions can be obtained from the authors. PCR products were purified by agarose gel electrophoresis and extraction from gel slices using a QiaGen kit. Approximately 200 ng of the PCR products were sequenced directly using 10 pmol of each PCR primer in the ABI DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) followed by automated analysis on an ABI Prism System 377. Sequence analysis and alignments were performed using the MACAW program (30).

Site-directed mutagenesis, cRNA synthesis and oocyte expression

Point mutations were introduced into the human CIC-1 cDNA (5) by recombinant PCR using Pfu DNA polymerase (Stratagene). In separate reactions, two fragments were amplified with overlapping primers which contained the desired mutation. The two fragments were joined in a second PCR amplification, digested with appropriate restriction enzymes and ligated into the hClC-1 cDNA in the vector pTLN (31) which contains Xenopus β-globin sequences to boost expression. All PCR-derived fragments were sequenced entirely. Plasmids were linearized with MluI and capped cRNA was transcribed using SP6 RNA polymerase in the Message mMachine cRNA synthesis kit (Ambion). cRNA (5–10 ng) was injected into Xenopus oocytes as described (32). Oocytes were kept in modified Barth’s solution (90 mM NaCl, 1 mM KCl, 1 mM CaCl2, 0.33 mM Ca(NO3)2, 0.82 mM MgSO4, 10 mM HEPES, pH 7.6) at 17°C for 2–3 days before measurement.

Electrophysiology

Standard two-electrode voltage-clamp measurements were performed at room temperature (20–22°C) in ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4) using a Turbotec amplifier (Npi Instruments) and pCLAMP 5.5 software (Axon Instruments). Apparent open probability (Po) was obtained with the pulse protocol shown in Figure 2. The membrane potential was first clamped to various test potentials ranging from −140 to +100 mV in steps of 20 mV. This was followed by a constant tail voltage of −80 mV. Extrapolated peak currents at this voltage were fitted using a Boltzmann distribution of the form

\[ I(V) = I_0 + (I_{\text{max}} - I_0)/(1 + \exp(V/V_1/2 - V/RT)) \]  

where \( I_{\text{max}} \) is the recorded current at maximal stimulation, \( z \) the apparent gating charge and \( V_1/2 \) the voltage of half-maximal activation. \( I_0 \) represents small (as compared with \( I_{\text{max}} \)), constant currents endogenous to the oocyte. Apparent \( P_0 \) was obtained by normalizing \( P_0 = I(V)/I_{\text{max}} \). Wild-type CIC-1 and all mutants were measured in at least two batches of oocytes (n ≥ 8 oocytes total).

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REFERENCES