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Molecular dissection of gating in the ClC-2 chloride channel

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The ClC-2 chloride channel is probably involved in the regulation of cell volume and of neuronal excitability. Site-directed mutagenesis was used to understand ClC-2 activation in response to cell swelling, hyperpolarization and acidic extracellular pH. Similar to equivalent mutations in ClC-0, neutralizing Lys566 at the end of the transmembrane domains results in outward rectification and a shift in voltage dependence, but leaves the basic gating mechanism, including swelling activation, intact. In contrast, mutations in the cytoplasmic loop between transmembrane domains D7 and D8 abolish all three modes of activation by constitutively opening the channel without changing its pore properties. These effects resemble those observed with deletions of an amino-terminal inactivation domain, and suggest that it may act as its receptor. Such a 'ball-and-chain' type mechanism may act as a final pathway in the activation of ClC-2 elicited by several stimuli.

Keywords: activation by pH/ball-and-chain/cell volume regulation/rectification/swelling activation

Introduction

ClC-2 (Thiemann *et al.*, 1992) belongs to a large gene family of chloride channels and putative chloride channels which is conserved in plants (Hechenberger *et al.*, 1996; Lurin *et al.*, 1996) and even in organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* (Greene *et al.*, 1993), and which includes at least nine different members in mammals (for reviews, see Jentsch *et al.*, 1995; Jentsch, 1996). Their physiological importance is best illustrated by human inherited diseases resulting from mutations in their genes. Thus, mutations in ClC-1, the major skeletal muscle chloride channel (Steinmeyer *et al.*, 1991) lead to recessive and dominant myotonia (Koch *et al.*, 1992; Pusch *et al.*, 1995b), and loss-of-function mutations in ClC-5 (Fisher *et al.*, 1995; Steinmeyer *et al.*, 1995) result in Dent's disease and other hereditary syndromes associated with kidney stones (Lloyd *et al.*, 1996).

The almost ubiquitously expressed ClC-2 is largely closed under resting conditions, but can be slowly activated by strong hyperpolarization (Thiemann *et al.*, 1992). ClC-2 has also been heterologously expressed in neuronal cells and again yielded typical inwardly rectifying currents (Staley *et al.*, 1996). Its drastic activation by osmotic cell swelling in the *Xenopus* oocyte expression system (Gründer *et al.*, 1992) suggests a role in regulating cell volume (Gründer *et al.*, 1992; Strange *et al.*, 1996), probably in parallel to other swelling-activated chloride channels with different biophysical characteristics (Worrell *et al.*, 1989; Solc and Wine, 1991; Jackson and Strange, 1995; Strange *et al.*, 1996). Expression of ClC-2 mRNA correlates with the presence of a hyperpolarization-activated chloride current with similar voltage dependence and kinetics as ClC-2 in neuronal (Chesnoy-Marchais, 1982; Madison *et al.*, 1986; Staley, 1994; Smith *et al.*, 1995) and epithelial cells and tissues (Komwatana *et al.*, 1994; Arreola *et al.*, 1996; Fritsch and Edelman, 1996), as well as in other cells (Chesnoy-Marchais and Fritsch, 1994). However, the halide selectivity which distinguishes ClC-2 currents from other hyperpolarization-activated anion currents (e.g. Kowdley *et al.*, 1994) has not been tested in many of these studies.

In neurons, the intracellular chloride concentration can be significantly below or above its electrochemical equilibrium. This determines whether opening of $GABA_A$ receptor chloride channels will be inhibitory, or, paradoxically excitatory. It has been proposed that expression of ClC-2 in certain neurons ensures an inhibitory response to GABA by preventing intracellular chloride from rising above its electrochemical equilibrium (Staley, 1994; Smith *et al.*, 1995). Indeed, ClC-2 mRNA is present in a subset of central neurons which display a hyperpolarization-activated chloride current and an inhibitory GABA response (Smith *et al.*, 1995), and transfection of ClC-2 into DRG neurons changes their GABA response from excitatory to inhibitory (Staley *et al.*, 1996). While activation by hyperpolarization occurs only at very negative (probably unphysiological) voltages in the *Xenopus* oocyte expression system (Thiemann *et al.* 1992), it activates significantly already at –50 mV in mammalian cells (Staley *et al.*, 1996). Thus, it is well suited to determine the intracellular chloride concentration.

Immunocytochemistry has revealed that ClC-2 is expressed in apical membranes of respiratory epithelial cells (Murray *et al.*, 1995), the same site where CFTR, the chloride channel defective in cystic fibrosis, is normally expressed. This makes ClC-2 an interesting target for therapy in cystic fibrosis, as activation of this largely dormant channel could provide an alternative pathway for the chloride secretion defective in that disease. It has not yet been proven that ClC-2 is involved in transepithelial transport; however, its localization in epithelia and activation by cell swelling (which may serve to couple it to transport processes across the opposite cell membrane) renders this into an exciting possibility.

A first important step towards understanding the mechanism of ClC-2 activation was the identification of a cytoplasmic, amino-terminal inactivation domain (Gründer *et al.*, 1992), the deletion of which leads to a

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constitutive activation with a complete loss of swelling- and voltage-sensitivity. When transplanted to the carboxy-terminus of the protein, this domain retained its effect on gating, indirectly suggesting a 'ball-and-chain'-type gating mechanism (Gründer *et al.*, 1992). In this model (Armstrong and Bezanilla, 1977), which is well supported for potassium channels (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990; Isacoff *et al.*, 1991), binding of an intracellular 'ball' (inactivation) domain to a 'receptor' on the channel backbone leads to its inactivation.

In this work we significantly advance our understanding of ClC-2 gating. We first show that ClC-2 is also opened by acidic extracellular pH, which operates via the common gating mechanism. This type of activation may be important in several physiological and pathophysiological settings. We then identify two novel regions in the channel protein which are important for gating. Mutating a lysine at the end of the last transmembrane span changes open channel rectification, and shifts the voltage dependence of gating. The basic gating mechanism, however, which includes the activation by cell swelling, is left intact. By contrast, mutations in the cytoplasmic loop between domains D7 and D8 abolish gating by constitutively opening the channel, which is now insensitive to swelling, hyperpolarization, and external pH. This is compatible with a role as a receptor for the amino-terminal, cytoplasmic inactivation domain, mutations in which have identical effects.

Results

ClC-2 can be activated by acidic extracellular pH

In addition to swelling and hyperpolarization, we now show that also acidic extracellular pH (pH_o) opens ClC-2 (Figure 1A and D). Sensitivity is high in the physiological range of pH_o and significantly modulates ClC-2 at resting potentials. Activation by pH reaches steady state within a few seconds, which is within the time range needed for a complete exchange of the bath solution. This suggests that the effect is due to external pH (pH_o), with pH_o primarily affecting gating. With extracellular acidification, and in contrast to neutral values of pH_o, currents are already activated at resting potentials of the oocyte (~–30 mV). This is evident from the instantaneous currents when stepping to the test voltages (Figure 1A). Further, steady-state currents (which still need several seconds to develop fully) are also increased at all voltages examined. This apparent opening of ClC-2 by acidic pH_o may be explained by a shift of its voltage dependence to more positive potentials. This is difficult to determine exactly, as voltage activation does not saturate even at –160 mV (oocytes do not tolerate more negative voltages), precluding reasonable fits with Boltzmann-type equations. Thus, we resorted to a changed pulse protocol in which the prepulse was shifted to more positive voltages. When this changed protocol was applied to channels 'activated' by acidic pH_o, the currents resembled wild-type (WT) currents with the normal protocol (Figure 1B). Thus, pH_o may act mainly by shifting the voltage dependence of ClC-2 gating.

As shown previously for the gating in response to hyperpolarization and cell swelling (Gründer *et al.*, 1992), the effect of pH_o also depends on the amino-terminal inactivation domain. Deleting amino acids 16 to 61 produces a constitutively open channel with a linear current–voltage (I–V) relationship (Gründer *et al.*, 1992), which is only marginally pH sensitive (Figure 1C and E). This may represent a slight pH-sensitivity of the open pore.

A lysine at the end of the last transmembrane span influences both gating and open channel rectification

To identify structures involved in gating distinct from the amino-terminal domain identified previously (Gründer *et al.*, 1992), we at first focused on K566, a lysine at the end of the transmembrane spanning domains. The reason for choosing this amino acid was 2-fold. First, in the *Torpedo* channel ClC-0, mutating the equivalent amino acid (K519) changes both pore properties and gating (Pusch *et al.*, 1995a; Ludewig *et al.*, 1996; Middleton *et al.*, 1996). Second, our previous experiments (Gründer *et al.*, 1992) were compatible with a ball-and-chain mechanism (Armstrong and Bezanilla, 1977), in which an amino-terminal inactivation domain (ball) binds to a cytoplasmic receptor on the channel backbone, thereby inhibiting (closing) the channel. In potassium channels, the putative receptor for the amino-terminal ball (the S4–S5 linker) is close to the pore, and mutations in this receptor can change pore properties (Isacoff *et al.*, 1991; Slesinger *et al.*, 1993). Thus, if K566 were close to the ClC-2 pore, it may also form part of a hypothetical receptor for the inactivation domain.

Similar to the mutation in ClC-0 (Pusch *et al.*, 1995a), neutralization of K566 (K566Q) resulted in an outward rectification of open channels, and reversing the charge (K566E) increased rectification further (Figure 2D and E). In contrast to ClC-0, however, anion selectivity was unchanged (data not shown). Compared with WT ClC-2 (Figure 2A), the mutant channel was significantly opened at resting potentials (Figure 2B), and a voltage–clamp protocol with a positive prepulse revealed that this was due to a large shift of its voltage dependence towards positive potentials (Figure 2C). Since voltage activation of ClC-2 is not complete even at –180 mV, this shift is difficult to quantify, but can be estimated to be ~80 mV.

Activation by hyperpolarization (Figure 2B and C), acidic pH_o (Figure 2F and G) and cell swelling (Figure 2H) was retained. The outward rectification of the open pore leads to outwardly rectifying currents with channels activated by acidic pH_o (Figure 2F). This also explains why swelling-activated K566Q channels, in contrast to swelling-activated WT ClC-2 (Gründer *et al.*, 1992), have a rather linear I–V relationship (Figure 2H).

Thus, similar to ClC-0 (Pusch *et al.*, 1995a), the lysine at the end of D12 is an important determinant of pore properties (rectification) and voltage-dependent gating. In contrast to amino-terminal deletions (Gründer *et al.*, 1992), it leaves the basic (and apparently ClC-2-specific) gating mechanism intact, including the activation by cell swelling. This suggests that it is not an important part of a hypothetical receptor for the amino-terminal inactivation domain.

Mutations in the cytoplasmic D7–D8 linker open ClC-2

Next we used a chimeric approach to search systematically for a cytoplasmic region in the channel protein whose

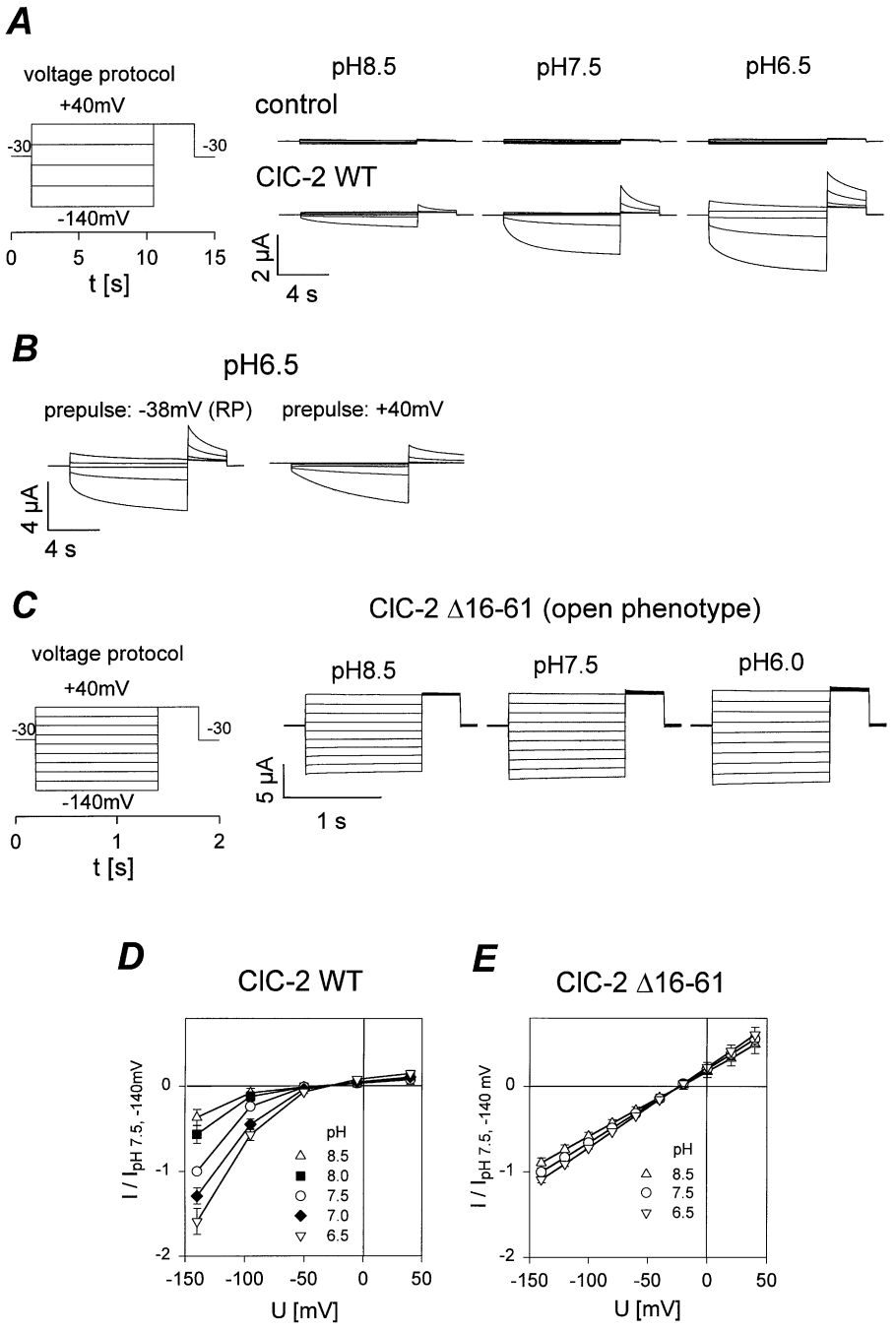


Fig. 1. Modulation of ClC-2 by extracellular pH. (**A**) Voltage–clamp traces from a control oocyte and a typical oocyte expressing rat ClC-2 after 1 min perfusion with ND96 at pH 8.5 (left), pH 7.5 (centre) and pH 6.5 (right). Voltage was clamped for 9 s each from a holding potential of –30 mV to values between +40 mV and –140 mV in steps of –45 mV, followed by a 3 s test pulse to +40 mV (shown at left). (**B**) ClC-2 currents activated by pH_o = 6.5 examined with two different pulse protocols. On the left [similar to (A)], a 20 s prepulse at the resting potential (–38 mV) precedes the 9 s test pulses (from +40 to –140 mV in steps of –45 mV); the channel is already significantly open at the resting potential, shown by the instantaneous currents when stepping to the test pulses. On the right, a positive prepulse (+40 mV) shows that this closes the channel, resulting in currents resembling ClC-2 at pH_o = 7.5. Thus, the effect of acidic pH_o may be explained by a shift in voltage dependence of gating. (**C**) pH dependence of the open phenotype amino-terminal gate deletion mutant ClC-2 Δ16–61 (Gründer *et al.*, 1992) after 1 min perfusion with ND96 at pH 8.5 (left), pH 7.5 (centre) and pH 6.0 (right). Voltage was clamped (left) for 1.2 s each from a holding potential of –30 mV to values between +40 mV and –140 mV in steps of –20 mV, followed by a 400 ms test pulse to +40 mV. (**D**) Steady-state I–V curves of wild-type ClC-2 at different values of extracellular pH (▽, pH 6.5; ◆, pH 7.0, ○, pH 7.5; ■, pH 8.0; △, pH 8.5). Channels open significantly with extracellular acidification. Experiments were performed as in Figure 1A and C and averaged results from five oocytes are shown. Currents were measured after 9 s, and normalized to currents at –140 mV and pH 7.5. (**E**) pH dependence of the 'open' ClC-2 Δ16–61 mutant (Gründer *et al.*, 1992) deleted for the amino-terminal inactivation domain [symbols as in (D)]. Only a slight pH dependence over the entire voltage range remains. Mean currents from four oocytes normalized to currents at –140 mV, pH 7.5 are shown.

mutation would lead, similar to deletions in the amino-terminus, to constitutively open channels. Within the framework of the ball-and-chain model for ClC-2, such a region would be a candidate receptor for the amino-terminal inactivation domain (Gründer *et al.* 1992).

ClC-1, the major skeletal muscle chloride channel

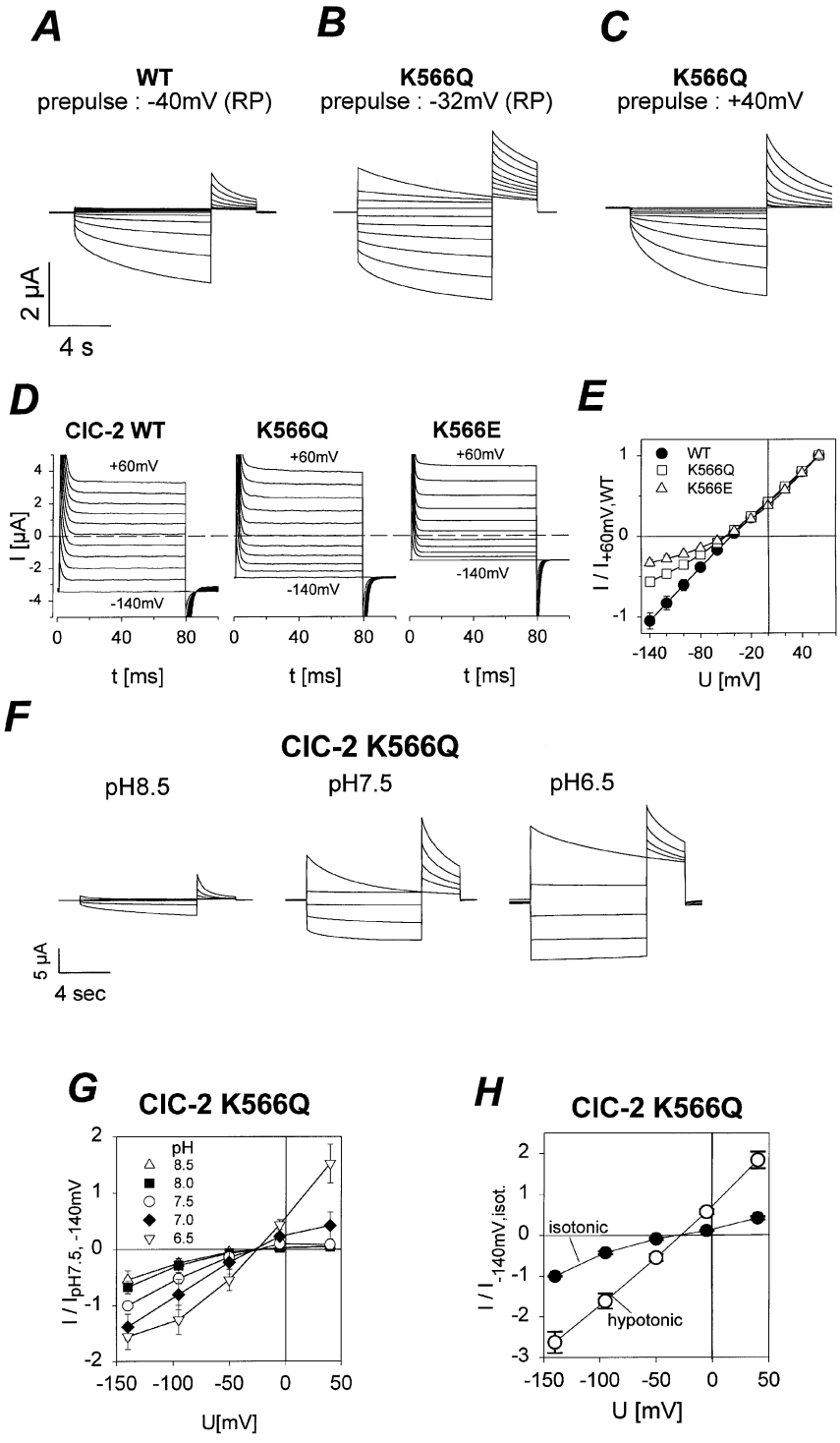
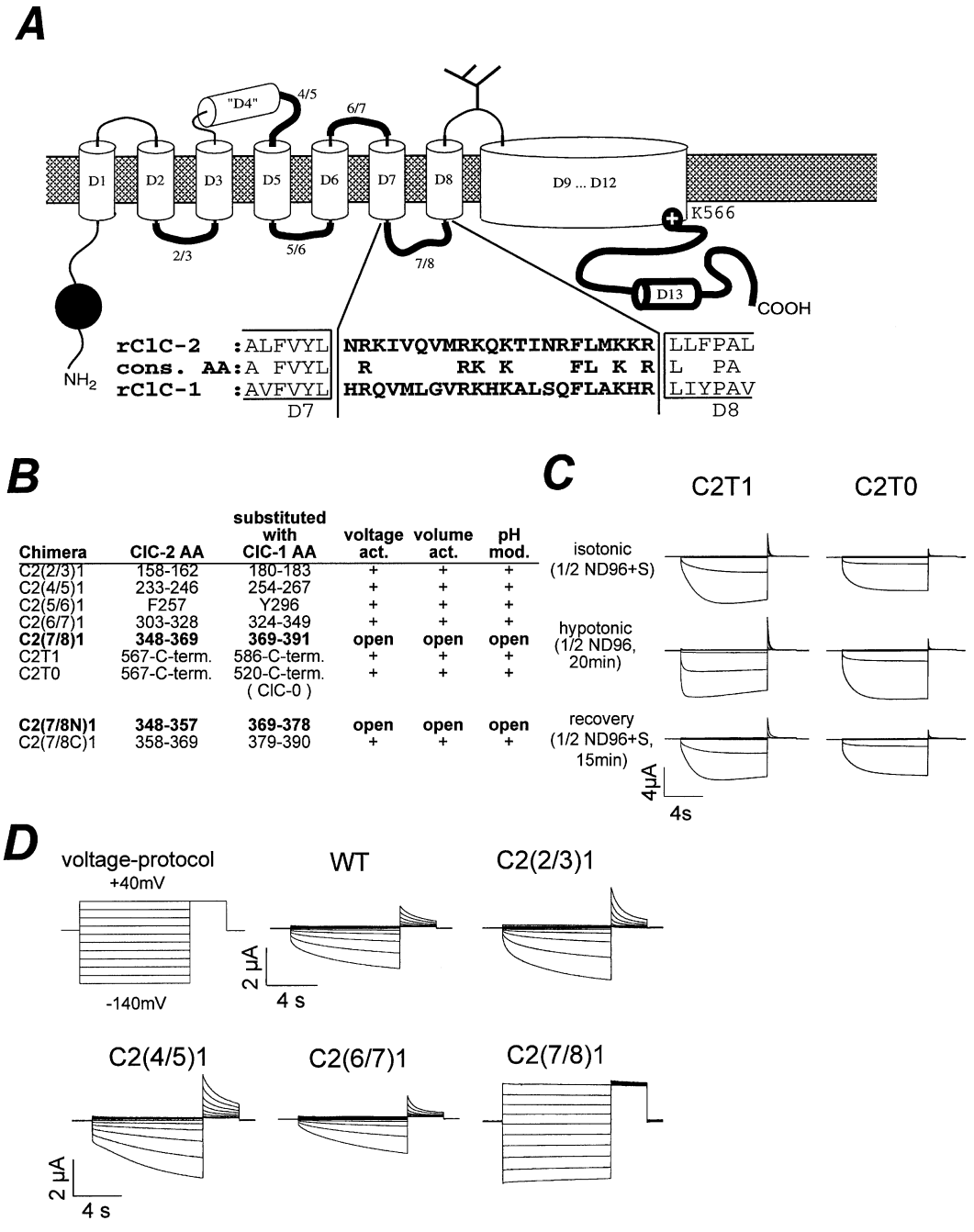


Fig. 2. Effects of mutating Lys566 at the end of the transmembrane spanning region. Voltage–clamp traces of wild-type ClC-2 (**A**) and its K566Q mutant (**B** and **C**) respectively, examined with the following pulse protocol: prepulse at the given voltage for 20 s, followed by 9 s pulses to voltages from +40 mV to –140 mV in steps of 20 mV, followed by a 3 s pulse to +40 mV. K566 is located at the end of the transmembrane spans (see topology model in Figure 3A). K566Q significantly opens the channel at the resting potential (RP) (B). When the mutant is held at a depolarizing voltage of +40 mV (C), its activation becomes similar to WT held at –40 mV (A), showing that the difference is largely due to a shift in the voltage dependence of gating. (**D**) Instantaneous currents of ClC-2 wild type, K566Q and K566E. Channels were activated by a single hyperpolarizing prepulse to –140 mV for 25 s, and currents measured during short (80 ms) pulses to voltages between +60 mV and –140 mV in steps of 20 mV. Normalized (to the current at +60 mV) currents averaged from five oocytes are shown in (**E**). In contrast to the linear I–V of WT ClC-2 (●), K566Q (□) and even more so K566E (△) are outwardly rectifying. (**F** and **G**), K566Q channels remain sensitive to extracellular pH. In (F), current traces of a typical K566Q expressing oocyte clamped according to the protocol of Figure 1A at three different values of external pH. With the shifted voltage dependence of K566Q, the mutant channel is more affected in this pH range than WT ClC-2 (Figure 1A). In (G), mean values of steady-state currents of five oocytes at five values of external pH as a function of voltage. △, pH 8.5; ■, pH 8.0; ○, pH 7.5; ◆, pH 7.0; ▽, pH 6.5. (**H**) K566Q channels still respond to osmotic swelling. Oocytes were measured in ND96 (●) and in half-ND96 after 20 min (○). Mean values of currents (measured after 9 s at the specified potential and normalized to the current at –140 mV in ND96) from four oocytes are shown.



Chimera	CIC-2 AA	substituted with CIC-1 AA	voltage act.	volume act.	pH mod.
C2(2/3)1	158-162	180-183	+	+	+
C2(4/5)1	233-246	254-267	+	+	+
C2(5/6)1	F257	Y296	+	+	+
C2(6/7)1	303-328	324-349	+	+	+
C2(7/8)1	**348-369**	**369-391**	**open**	**open**	**open**
C2T1	567-C-term.	586-C-term.	+	+	+
C2T0	567-C-term.	520-C-term. (ClC-0)	+	+	+
C2(7/8N)1	**348-357**	**369-378**	**open**	**open**	**open**
C2(7/8C)1	358-369	379-390	+	+	+

Fig. 3. Identification of the putative intracellular receptor for the amino-terminal inactivation domain. (**A**) Chimeric strategy. Extramembranal segments of rClC-2 were replaced by the corresponding ones from rClC-1, which is not influenced by the ClC-2 amino-terminus (Gründer *et al.*, 1992). The substituted segments are shown with bold lines in the current topology model of ClC channels (Jentsch, 1996). Since the topology is largely conjectural with the exception of the glycosylated loop after D8 (Kieferle *et al.*, 1994; Middleton *et al.*, 1994), some putative extracellular loops were also substituted. The amino-terminal inactivation domain (ball) characterized in Gründer *et al.* (1992) is shown as a filled circle. The sequence of the putative receptor, the intracellular loop between D7 and D8, is shown below in comparison to rClC-1 with which it was substituted in chimera C2(7/8)1. (**B**) List of chimeras and their phenotypes. The numbers in brackets indicate which loop is replaced by the corresponding ClC-1 sequence. In C2T1 and C2T0, the entire cytoplasmic tail is replaced by that of ClC-1 and ClC-0, respectively. Columns 2 and 3 give the numbers of amino acids which were substituted, and the next three columns indicate whether the mutants were still activated by hyperpolarization, swelling or acidic pH, respectively. The last two lines give results for chimeras in which only the amino- or carboxy-terminal halves of the putative receptor loop were exchanged. (**C**) Voltage- and cell-swelling-dependent gating of the carboxy-terminal chimeras C2T1 and C2T0. The voltage dependence of gating and the sensitivity to extracellular osmolarity (and pH, not shown) are retained, but swelling mainly increases the rate of voltage activation, and not steady-state currents. Representative traces are shown from an oocyte injected with chimeric cRNA incubated in ND96 (top), hypotonic half-ND96 (middle), and after recovery in ND96 (bottom). Voltage steps from +20 mV to –160 mV for 3 s each, followed by a pulse to +40 mV for 1 s. (**D**) Voltage–clamp traces for the individual chimeras expressed in *Xenopus* oocytes, and the voltage–clamp protocol (top left). Except for the loop between domains D7 and D8, all chimeras (including a complete exchange of the carboxy-terminus) qualitatively retained the sensitivity to voltage, pH, and swelling.

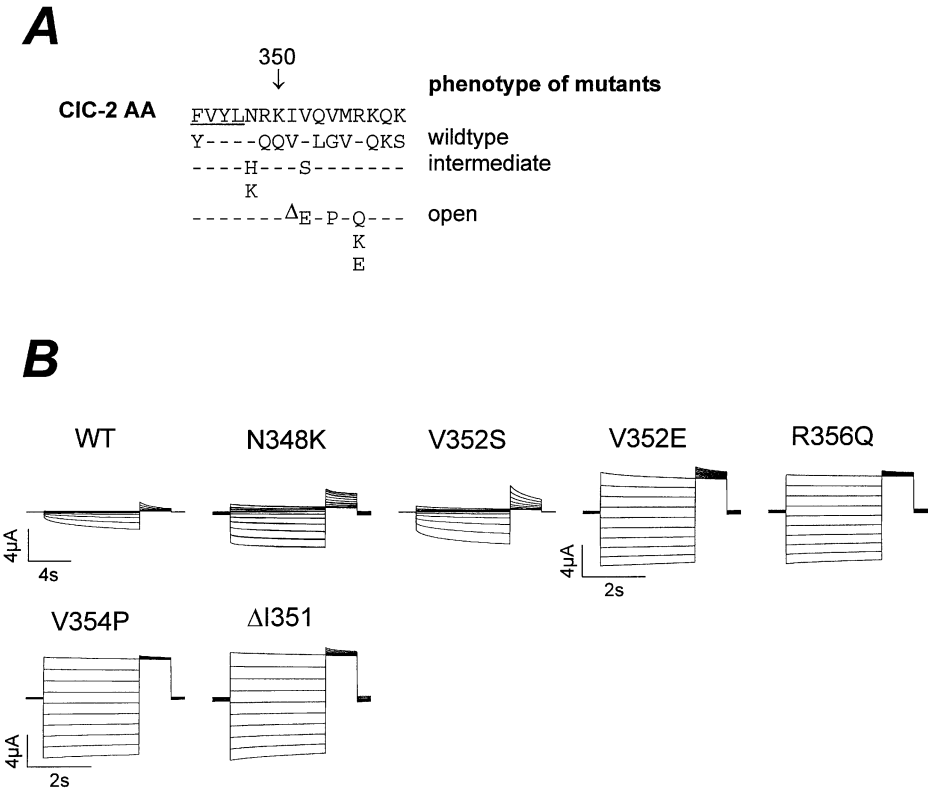


Fig. 4. Analysis of point mutations in the putative receptor between domains D7 and D8. (**A**) Positions of point mutations in the amino-terminal part of the loop sequence. Chimera C2(7/8C)1 (Figure 3B) indicates that the carboxy-terminal part is less important. The end of the putative transmembrane domain D7 is underlined. Amino acids were exchanged singly, and shown in lanes according to their resulting phenotype (wildtype, intermediate or open). Amino acids were mostly mutated to the corresponding residues present in ClC-1. Intermediate mutations were partially open, similar to some mutations in the amino-terminus (Gründer *et al.*, 1992). (**B**) Voltage–clamp traces of WT ClC-2 and point mutants in the putative receptor loop. Voltage–clamp protocol as in Figure 2C, but shorter pulses with open mutants. N348K and V352S display an 'intermediate', partially open phenotype, while the other mutants shown are open mutants.

(Steinmeyer *et al.*, 1991), is 55% identical to ClC-2 (Thiemann *et al.*, 1992). As ClC-1 currents are unaffected by the ClC-2 amino-terminus (Gründer *et al.*, 1992), we reasoned that ClC-1 lacks such a hypothetical receptor. We therefore systematically replaced loops between ClC-2 transmembrane spans and the cytoplasmic carboxy-terminus with those from ClC-1 (Figure 3A). As the exact topology of ClC-channels is still unclear, we also chose loops located extracellularly in the current topology model (Jentsch, 1996). However, we excluded the loop between D8 and D9 as its glycosylation in ClC-0, ClC-1, ClC-2 and the ClC-K channels (Kieferle *et al.*, 1994; Middleton *et al.*, 1994) prove its extracellular localization.

Most replacements resulted in currents which retained activation by swelling, hyperpolarization and acidic pH_o. When the entire large carboxy-terminus of ClC-2 was replaced by those of ClC-1 and ClC-0 in the chimeras C2T1 and C2T0, respectively, activation by hyperpolarization and pH_o were retained; currents were still sensitive to swelling, but its effect was less pronounced (Figure 3C). With C2T1, swelling predominantly increased the rate of voltage activation (Figure 3C).

By contrast, the substitution of the intracellular loop connecting transmembrane domains D7 and D8 specifically yielded constitutively open channels (Figure 3B and D) which were no longer sensitive to voltage, pH and swelling. The D7–D8 linker, though always highly positively charged, is poorly conserved between different ClC members. In contrast, there are only a few amino acid changes in the D2–D3 and D5–D6 linkers between ClC-1 and ClC-2. By studying partial replacements of the D7–D8 linker, the important region was narrowed down to the first half of that stretch (Figure 3B).

Site-directed mutagenesis demonstrated that even single point mutations could open the channel (Figure 4). Mutations of an asparagine residue (N348 to histidine or lysine), directly after putative transmembrane domain D7, caused a channel with an intermediate phenotype. Instantaneous currents were larger than those of WT, resembling a volume-activated channel. Substitution of a valine residue (V352) with serine also produced an intermediate phenotype with reduced voltage-sensitivity. Mutating this residue to glutamate (V352E) completely opened the channel, making it insensitive to voltage, medium tonicity and pH. Mutations of an arginine residue (R356, to glutamine, glutamate or lysine) had the same profound effect on gating. The distance between critical residues (N348, V352 and R356) is compatible with a helical structure of that segment, as they would face the same side of a helix. This was supported by mutations disrupting the secondary structure of that segment by either deleting an amino

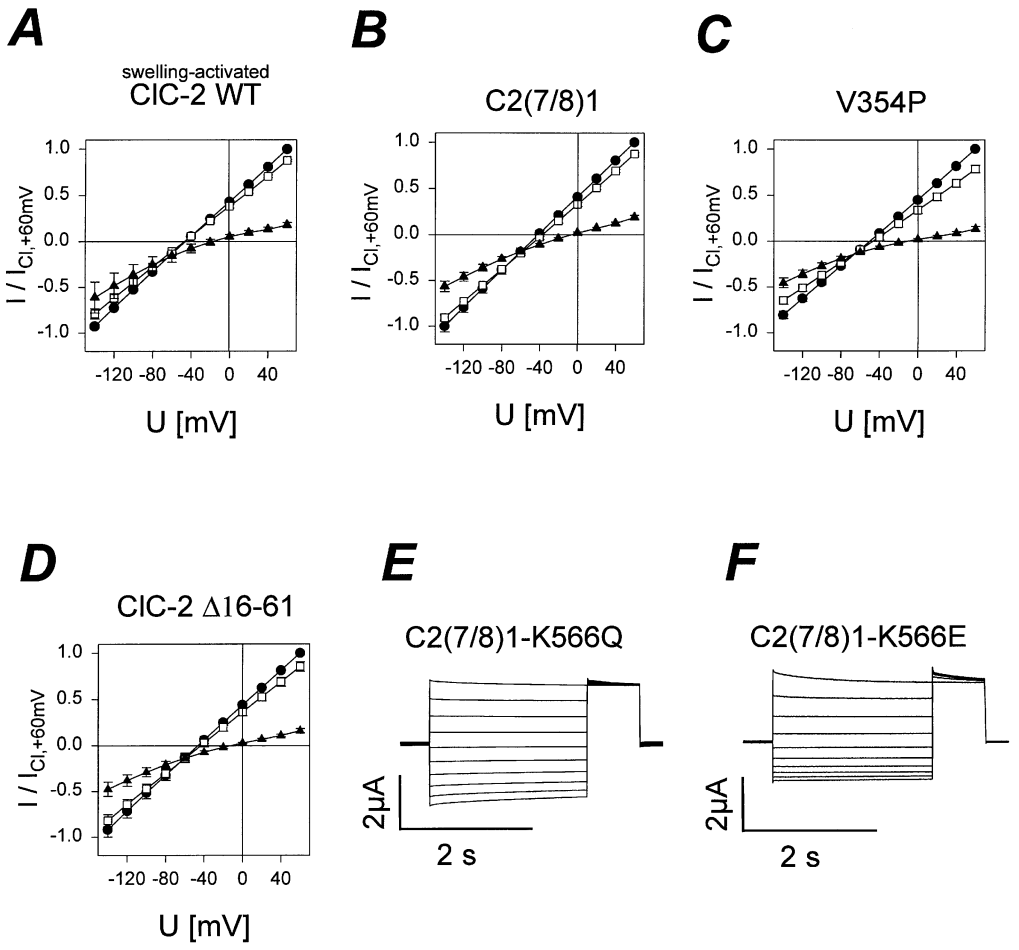


Fig. 5. Pore properties of WT ClC-2 and mutants abolishing the ball-and-chain activation mechanism. Halide selectivity of instantaneous currents of (**A**) ClC-2 activated by cell swelling (average of four cells), (**B** and **C**) ClC-2 activated by destroying the putative receptor [mutants C2(7/8)1 and V354P, cf. Figure 3] (average of four cells), and (**D**) ClC-2 activated by deleting the ball domain (mutant Δ16–61; Gründer *et al.*, 1992) (average of seven cells); ●, 96 mM NaCl; □, 96 mM NaBr; ▲, 96 mM NaI. For the channel activated by swelling (A), measurements were made in 48 mM NaCl, 48 M NaBr and 48 mM NaI, respectively (half-osmolarity). Voltage protocols are as in Figure 2E, but for the open mutants (B,C,D) the initial 25 s activation by hyperpolarization was omitted. All currents were normalized to the currents at +60 mV in ND96. Halide selectivity was also checked and found unchanged in mutants N348K, V352S, 352E and R356Q (data not shown). (**E** and **F**) Rectification of ClC-2 mutants opened by additional mutations in the receptor. In contrast to WT channels opened by receptor mutations, which have a linear I–V (Figure 4B), combinations with the K566Q (E) or K566E (F) mutations yield outwardly rectifying currents. These are similar to the instantaneous currents of channels carrying these point mutations alone (cf. Figure 2G). Voltage protocol as in Figure 2C, with shorter pulses.

acid (ΔI351) or by introducing a helix-breaking residue (V354P).

Mutations in the amino-terminal inactivation domain and the D7–D8 linker have overriding effects on gating, but do not affect the pore properties influenced by K566

Channels opened by mutations in the amino-terminal inactivation domain or in the D7–D8 loop retain the pore properties of the starting construct. Thus, they share the typical halide selectivity (Cl>Br>I) (Figure 5B–D) with WT ClC-2 activated by swelling (Figure 5A) or hyperpolarization (Thiemann *et al.*, 1992). Further, when starting from WT ClC-2, their I–V is almost linear, irrespective of whether the amino-terminus (Figure 1E) or the D7–D8 loop (Figures 3D, 4B and 5B–D) is mutated. This corresponds to the approximately linear I–V of instantaneous ClC-2 currents (Thiemann *et al.*, 1992; Figure 2E). When these mutations are combined with the K566Q or the K566E mutations, the resulting channels have an outwardly rectifying I–V (Figure 5E and F), which also corresponds to the instantaneous currents of single mutants in this residue (Figure 2D and E). Thus, both types of mutations abolishing the activation process do not interfere with the pore properties of ClC-2.

Discussion

In this work, we have identified two distinct regions in the ClC-2 chloride channel protein, which, in addition to the amino-terminal region identified previously (Gründer *et al.*, 1992), are important for the opening of the channel. While mutations at the end of the last transmembrane span quantitatively affect gating (and permeation), mutations in the cytoplasmic loop between transmembrane spans D7 and D8 abolish gating by constitutively activating the channel. This is compatible with a role as a receptor for the amino-terminal inactivation domain identified previously, and suggests that a ball-and-chain mechanism of activation may be the common final pathway for several types of

stimulation. This includes a novel activation by acidic extracellular pH.

Differentiation from endogenous oocyte channels

Great care has to be taken when chloride channels are expressed heterologously in *Xenopus* oocytes or other cells. *Xenopus* oocytes express many different endogenous chloride channels in their plasma membrane. This includes Cl^--channels activated by intracellular Ca^{2+} (Miledi, 1982; Boton *et al.*, 1989), channels activated by removal of extracellular divalent cations (Weber *et al.*, 1995), swelling-activated channels (Ackerman *et al.*, 1994), hyperpolarization-activated channels (Parker and Miledi, 1988; Kowdley *et al.*, 1994) and outwardly rectifying channels (Steinmeyer *et al.*, 1995). For some of these channels, expression is highly variable between different batches of oocytes, and some can be activated (quite unspecifically) by overexpressing foreign proteins.

The endogenous hyperpolarization-activated anion current of oocytes (Kowdley *et al.*, 1994) superficially resembles the currents induced by ClC-2: both are activated with a similar slow time-course by hyperpolarizations to similar voltages. Very similar currents can be induced by overexpressing several membrane proteins in oocytes (Moorman *et al.*, 1992; Shimbo *et al.*, 1995; Tzounopoulos *et al.*, 1995), including otherwise inactive ClC proteins (Steinmeyer *et al.*, 1994; Brandt and Jentsch, 1995). However, these endogenous currents clearly differ from ClC-2 currents: First, they activate transiently when clamped to a positive (+40 mV) potential after activation by hyperpolarization (Kowdley *et al.*, 1994; our unpublished observations), while ClC-2 currents deactivate immediately (e.g. Figures 1 and 2). Second, endogenous currents are insensitive to external acidification (Kowdley *et al.*, 1994), which dramatically activates ClC-2 (Figure 1). Most importantly, however, ClC-2 has a Cl>I selectivity, while endogenous channels conduct iodide better than chloride. To preclude a contamination by these endogenous channels, we discarded batches of oocytes expressing these currents, injected only moderate amounts of cRNA to avoid overexpression, and additionally tested the halide selectivity in many experiments (Figure 5A–D).

Oocytes also express endogenous swelling-activated chloride currents, which depend on the follicular cell layer enclosing the oocyte (Ackerman *et al.*, 1994; Arellano and Miledi, 1995). These currents decay within 2 days after defolliculation. Hence, they were absent in the oocytes used in this work, which we confirmed by control measurements. Moreover, endogenous swelling-activated channels again have an I>Cl selectivity, and their voltage dependence differs markedly from those of swelling-activated ClC-2 channels.

Our experiments strongly suggest that the currents induced by ClC-2 are directly mediated by this channel protein. Much more convincing than a comparison with known oocyte channels is the fact that many mutations very specifically and in part drastically changed channel properties, including pore properties such as open channel rectification. In addition, heterologous expression of ClC-2 in DRG neurons (Staley *et al.*, 1996) and in CHO cells (our unpublished data) demonstrates that it does not need an oocyte-specific cofactor.

Activation by extracellular pH

In this work, we describe a novel activation of ClC-2 by extracellular pH. Sensitivity is maximal in the physiological pH range, and acidification significantly opens ClC-2 at normal resting potentials (–50 to –90 mV). Amino-terminal deletion mutants indicate that this is not due to a pH-sensitivity of the open pore, but via a modulation of a common gating mechanism also used by hyperpolarization and cell swelling. pH_o mainly modulates the voltage dependence of gating, which is apparently shifted to more positive voltages by acidification. This leads to an increase of currents at resting membrane potentials. Such an activation by acidic pH_o may be important in pathological settings like hypoxia, where opening of ClC-2 channels may counteract cell swelling. It may be crucial in cells facing acidic fluids, such as those in the distal nephron or stomach.

Interestingly, rabbit ClC-2 was proposed to be the cAMP-activated Cl^--channel involved in gastric acid secretion, and was therefore dubbed ClC-2G (G for gastric) (Malinowska *et al.*, 1995). However, just like its rat (Thiemann *et al.*, 1992) and human (Cid *et al.*, 1995) counterparts, it is not stomach-specific, but rather ubiquitously expressed (Furukawa *et al.*, 1995). Rat ClC-2 lacks consensus sites for cAMP-dependent phosphorylation, but such sites are present (at different positions) in the long cytoplasmic carboxy-terminus of both rabbit and human ClC-2. This carboxy-terminal stretch is variable even between ClC-0 channels from the closely related species *Torpedo marmorata* (Jentsch *et al.*, 1990) and *Torpedo californica* (O'Neill *et al.*, 1991). The PKA-sites in rabbit ClC-2 are thought to be functionally important (Malinowska *et al.*, 1995). When rabbit ClC-2 cRNA was injected into *Xenopus* oocytes and their membranes fused to lipid bilayers, Malinowska *et al.* detected single Cl^--channels activated by PKA. By contrast, we tested human ClC-2 (which has a similar PKA site) directly in the oocyte system, and could not detect any effect of raising intracellular cAMP to values sufficient to activate CFTR (data not shown). In human T84 cells, a native Cl^--current resembling ClC-2 is rather inhibited by cAMP (Fritsch and Edelman, 1996). It is surprising that the selectivity (I>Cl) of the reconstituted channel (Malinowska *et al.*, 1995) differs from the selectivity of ClC-2 (Cl>I) determined directly in the oocyte expression system (Thiemann *et al.*, 1992; Lorenz *et al.*, 1996; also this work).

Mutations in K566 and in the D7–D8 linker have qualitatively different effects on gating

In our search for regions important for gating, we first mutated K566 because mutations of the equivalent amino acid (K519) in the *Torpedo* channel ClC-0 affect both gating and permeation (Pusch *et al.*, 1995a). Indeed, with ClC-2, the effects were quite similar: voltage-dependent gating was shifted by several tens of millivolts to positive potentials, and the open channel I–V relationship was changed from slightly inwardly rectifying to outwardly rectifying. In analogy to previous results with the acetylcholine receptor (Imoto *et al.*, 1988), the outward rectification suggests that K566 is located next to the cytoplasmic entrance of the pore, and that the effect may be electrostatic. Indeed, a charge reversal (K566E) led to a more pronounced outward rectification than its neutralization

(K566Q). Also in ClC-0, mutations of this lysine led to a shift in the voltage dependence of 'fast' gating to the right (Pusch *et al.*, 1995a). However, it is presently unclear how the slow, hyperpolarization-activated gating of ClC-2 relates to the depolarization-activated fast gating of ClC-0. None the less, these data suggest that the lysine at the end of D12, even though not universally conserved, may be of more general importance in determining permeation and gating of ClC channels.

Importantly, mutations in K566 only modified, but did not abolish, gating of ClC-2. Thus, activation by hyperpolarization, cell swelling and acidic pH were retained. This is in contrast to mutations in the amino-terminal inactivation domain (Gründer *et al.*, 1992) and in the D7–D8 linker investigated here. Mutations in both these regions have identical effects: they constitutively open the channel which, at least in the range investigated, is now insensitive to voltage, cell swelling and acidic pH. This effect on gating overrides those of mutations in K566. In contrast to mutations in this lysine, pore properties are not affected by mutations in either region and, when combined with K566 mutants, their effects on open channel rectification are retained.

Even single point mutations in the D7–D8 loop could open the channel and their positions are compatible with an α-helical structure of this stretch. In contrast to the first preliminary topology model for ClC-0 (Jentsch *et al.*, 1990), the new topology model for ClC channels (Jentsch *et al.*, 1995) suggests that this loop is intracellular. This is supported by the glycosylation of the D8–D9 linker (Kieferle *et al.*, 1994; Middleton *et al.*, 1994), and the hydrophobicity analysis which indicates that D8 is a very good candidate for a transmembrane span (Brandt and Jentsch, 1995). In addition, a cytoplasmic position of this highly positively charged segment satisfies the 'positive-inside' rule for membrane proteins (von Heijne, 1992).

In contrast to exchanges of the amino-terminus (Gründer *et al.*, 1992), replacements of the large cytoplasmic carboxy-terminus of ClC-2 by those of ClC-0 or ClC-2 left the basic gating mechanism intact. This includes the sensitivity to cell swelling, which, however, was changed quantitatively. This may not be too surprising as more than one-third of the protein was replaced by mostly non-homologous sequences. This indicates that the carboxy-terminus plays some role in swelling-activation, and one could hypothesize that it may interact with cytoskeletal elements. Such an interaction, which conceivably could differ between these channels, is thought to be important in transducing cell swelling to channel opening (see e.g. Schwiebert *et al.*, 1994).

A gating model of the ball-and-chain with an activation influenced by pore structures

Gating of ClC-2, whether stimulated by hyperpolarization, swelling or acidic pH, can be abolished by mutations in either an amino-terminal, cytoplasmic inactivation domain, or in the cytoplasmic loop between transmembrane spans D7 and D8. The amino-terminal domain could be transplanted to the (also cytoplasmic) carboxy-terminus without loss-of-function (Gründer *et al.*, 1992), demonstrating that its effect is largely position-independent. This suggests that it inhibits channel opening by binding to another, cytoplasmic region of the channel protein. This region may well be the D7–D8 loop (or may have this loop as one component), as mutations in this cytoplasmic stretch have effects identical to those observed with mutations in the amino-terminal inactivation domain. Thus, within the ball-and-chain model, this loop may be the receptor for the inactivation domain (ball). However, preliminary experiments in which we transplanted both the amino-terminus and the D7–D8 linker to the ClC-1 channel did not confer hyperpolarization- or swelling-induced gating on its currents (data not shown). This could mean that other structures specifically present in ClC-2 are needed for forming a functional receptor, or that ClC-2 specific structures are needed to couple a hypothetical ball–receptor interaction to channel opening. It is also interesting to note that ClC-1/ClC-2 co-expression yields currents which may be explained by a double-barrelled channel with one ClC-1 and one ClC-2 pore, the latter having lost its voltage- and swelling-dependence (Lorenz *et al.*, 1996). We clearly cannot exclude that the D7–D8 linker does not physically interact with the ball, and that mutations in this segment open the channel by an unknown mechanism.

We envisage a model in which hyperpolarization, swelling and acidic pH act by a final common pathway, possibly according to the ball-and-chain model. These stimuli would lead to a reduced affinity of the receptor for the ball, whose dissociation would then lead to an opening of the channel. As shown by mutations in K566, structures related to the pore are important in conveying the effect of hyperpolarization. How this works in detail is unclear, but is vaguely reminiscent of ClC-0, where permeation and gating are intimately linked (Pusch *et al.*, 1995a).

In potassium channels, mutations in the S4–S5 linker, an important part of the putative receptor for the inactivation particle, can change pore properties (Isacoff *et al.*, 1991; Slesinger *et al.*, 1993). Thus, the close proximity of the receptor with the pore is compatible, in principle, with the simple picture of the ball 'plugging' the pore. With ClC-2 this scenario seems less likely as pore properties were unaffected by mutations in the D7–D8 loop. There may well be a conformational change of the protein induced by the binding of the ball, which ultimately leads to a closure of the channel.

An exciting possibility is that alternative splicing of the ClC-2 amino-terminus could lead to constitutively open channels. Apparently, such a variant was found in a heart cDNA library (Furukawa *et al.*, 1995). It predicts a ClC-2 protein with a deleted inactivation domain, and accordingly its expression yielded constitutively open channels. However, the new 5′ sequence (35 bp) replacing the inactivation domain is highly homologous to sequences at the very ends of many sequenced cDNAs in the database, including over 50 clones from a rice genome project, and may represent a concatamer of three linkers (T.J.Jentsch, unpublished observation). Thus, it may be questioned whether it represents a splice variant found in cells.

Interference with the activation mechanism described here will offer possibilities to open ClC-2 pharmacologically. In ClC-0, permeation and gating is tightly coupled (Pusch *et al.*, 1995a), and external anions drastically affect channel open probability. The effects of the ClC-2 K566Q mutant (equivalent to the mutation studied for ClC-0) suggests that ClC-2 gating may have common properties.

Thus, it could even be possible to develop ClC-2 openers which act from the extracellular side by targeting the pore and changing the voltage dependence of ClC-2. An activation of the otherwise largely closed ClC-2 may be of benefit in conditions such as cystic fibrosis, in which ClC-2 conceivably could functionally substitute for CFTR, or in brain oedema, where activation of a chloride channel could counteract cell swelling.

Materials and methods

Construction of chimeras and site-directed mutagenesis

Defined segments from rat ClC-1 (Steinmeyer *et al.*, 1991) were introduced into the rat ClC-2 (rClC-2) cDNA (Thiemann *et al.*, 1992) by recombinant PCR using Pfu DNA-polymerase (Stratagene). Briefly, rClC-1 cDNA was amplified with two chimeric primers encompassing the desired rClC-1 sequence to be introduced into rClC-2 and containing terminal overlapping rClC-2 sequences. Two flanking rClC-2 sequences were amplified in separate reactions. All three sequences were joined by recombinant PCR, digested with appropriate restriction endonucleases, and ligated into the cut ClC-2/vector construct. PCR-derived fragments were entirely sequenced. In the chimera C2H1 the carboxy-terminus of rClC-1 (beginning at its native *Hin*dIII site) was cloned into rClC-2 via a silent *Hin*dIII introduced into rClC-2 at the equivalent position. The cDNAs were inserted into the vector PTLN (Lorenz *et al.*, 1996) which contains *Xenopus* β-globin sequences to boost expression (Krieg and Melton, 1984). Point mutations were introduced into rClC-2 cDNA as described by Pusch *et al.* (1995b).

cRNA synthesis and electrophysiology

Capped cRNA was transcribed by SP6 RNA polymerase from 0.5 μg plasmid DNA after linearization with *Mlu*I using the mMessage mMachine cRNA synthesis kit (Ambion). Portions of 2–10 ng cRNA (corresponding to 40–200 ng/ml) were injected into manually defolliculated *Xenopus* oocytes. Oocytes were kept at 16°C in modified Barth's solution (88 mM NaCl, 2.4 mM $NaHCO_3$, 1.0 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 10 mM HEPES, pH 7.6) for 3 days and analysed in ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). To determine halide selectivity, currents were measured in ND96, or in ND96 in which 96 mM Cl^- was either substituted by equivalent amounts of Br^- or I^-. Hypoosmotic stimulation was examined 20–30 min after replacing ND96 with half-ND96. In the pH modulation measurements, ND96 was buffered with 5 mM MES for pH 6.0 and 6.5, with 5 mM HEPES for pH 7.0 and 7.5 and with 5 mM Tris for pH 8.0 and 8.5. Standard two-electrode voltage–clamp measurements were performed at room temperature (20–22°C) using a Turbotec amplifier (Npi Instruments) and pCLAMP 5.5 software (Axon Instruments).

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