Cloning and Functional Expression of a Plant Voltage-Dependent Chloride Channel

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Plant cell membrane anion channels participate in basic physiological functions, such as cell volume regulation and signal transduction. However, nothing is known about their molecular structure. Using a polymerase chain reaction strategy, we have cloned a tobacco cDNA (CIC-N1) encoding a 780-amino acid protein with several putative transmembrane domains. CIC-N1 displays 24 to 32% amino acid identity with members of the animal voltage-dependent chloride channel (CIC) family, whose archetype is CIC-0 from the Torpedo marmorata electric organ. Injection of CIC-N1 complementary RNA into Xenopus oocytes elicited slowly activating inward currents upon membrane hyperpolarization more negative than −120 mV. These currents were carried mainly by anions, modulated by extracellular anions, and totally blocked by 10 mM extracellular calcium. The identification of CIC-N1 extends the CIC family to higher plants and provides a molecular probe for the study of voltage-dependent anion channels in plants.

INTRODUCTION

In resting plant cells, the cytoplasmic side of the vacuolar and plasma membranes is negatively polarized. Because of this polarization, the high concentration of anions in the cytoplasm has to be maintained by energy-dependent transport systems (Felle, 1994). In addition, anion channels in these membranes can mediate large passive fluxes of anions into the apoplasm or the vacuole. These fluxes, which can trigger a rapid shift of the membrane potential, participate not only in signal transduction but also in solute compartmentation and osmotic regulation of the cells (Ward et al., 1995).

Electrophysiological analyses have revealed the presence of anion channels in most of the plant cell membranes investigated. In addition to the ubiquitous slow activation vacuolar-type channel that has been described as somewhat permeable to anions (Hedrich and Becker, 1994), highly selective anion channels have been identified in the tonoplast of higher plants (Iwasaki et al., 1992; Pantoja et al., 1992; Cerana et al., 1995). A variety of anion channels that are constitutively active or activated by membrane depolarization, hyperpolarization, or stretch have also been described at the plasma membrane of higher plant cells (for reviews, see Hedrich and Becker, 1994; Schroeder, 1995). In particular, a detailed analysis of depolarization-activated anion channels at the plasma membrane of broad bean guard cells has suggested a role for these channels in the salt efflux that mediates stomatal closing (Hedrich and Becker, 1994; Ward et al., 1995). Similar voltage-dependent anion channels have also been described in protoplasts from tobacco cell suspension cultures (Zimmerman et al., 1994) and from epidermal cells of Arabidopsis hypocotyls (Thomine et al., 1995).

The molecular structure of many plant membrane channels and transporters has been elucidated recently; however, nothing is known about the structure of plant anion channels. This contrasts with the different classes of anion channel proteins that have been identified in animals (Paulmich et al., 1993). Among these, the family of voltage-dependent chloride channels or CIC family has received much attention (Jentsch et al., 1995). CICs are involved in a variety of cellular functions, such as the stabilization of trans-plasma membrane electrical potential (CIC-0, Jentsch et al., 1990; CIC-1, Steinmeyer et al., 1991), cell volume regulation (CIC-2, Gründer et al., 1992), or transcellular chloride transport (CIC-K1, Uchida et al., 1993; CIC-K2, Adachi et al., 1994). CIC-0, the first member of the CIC family, was cloned from the Torpedo marmorata electric organ by expression in Xenopus oocytes (Jentsch et al., 1990). The cloning of CIC-0 allowed the subsequent discovery of many CIC-related proteins, whose function was characterized mostly using oocyte expression. However, the channel function of CIC-0 was confirmed by reconstitution experiments with the purified protein in artificial membranes (Middleton et al., 1994; Rosenthal and Guidotti, 1994).

Recently, a homolog of the CIC family, named GEF1, was described in yeast (Greene et al., 1993). Mutations in the GEF1 gene cause cells to grow slowly on rich medium containing carbon sources utilized by respiration. This phenotype is suppressed by adding high concentrations of iron to the growth medium, suggesting that the GEF1 gene is required for iron metabolism during growth on respired carbon sources (Greene et al., 1993).
et al., 1993). The molecular activity of GEF1 in relation to this phenotype remains to be elucidated. Nevertheless, these results indicate that CIC genes are present in a large variety of living organisms and prompted us to search for putative CIC homologs in higher plants. Our strategy has been to use polymerase chain reaction (PCR), relying on the sequence homology of the animal and yeast CIC genes. In this study, we describe the isolation of CIC-Ntl from tobacco. CIC-Ntl is a new member of the CIC gene family. Its expression in Xenopus oocytes elicited inward chloride currents upon membrane hyperpolarization, and we propose that CIC-Ntl functions as a voltage-dependent chloride channel in plant membranes.

RESULTS

Cloning of a Tobacco CIC Homolog

A PCR cloning strategy was designed using two amino acid motifs highly conserved within the animal and yeast CIC homologs. A pair of degenerate primers permitted the amplification of a 197-bp cDNA fragment from cultured tobacco cells. The sequence of this fragment indicated that it represented a putative new member of the CIC family. RNA gel blot hybridization analysis of tobacco mRNA with the PCR product as a probe

Figure 1. Nucleotide and Deduced Amino Acid Sequences of CIC-Ntl.

Initiation and stop codons are underlined. Potential glycosylation sites are indicated by dots; potential phosphorylation sites for animal protein kinases A and C are noted by asterisks; and PCR primers used for amplification are double underlined. The sequence data for CIC-Ntl has EMBL accession number X95576.
confirmed that this fragment was indeed amplified from plant cDNA. This analysis further showed that the corresponding gene was expressed, although at varying levels, throughout all the plant organs, with the highest expression level in stems (data not shown).

High-stringency screening of a tobacco plantlet library with the PCR product as a probe yielded a 2610-bp cDNA clone that displayed 100% sequence identity with the PCR product. The length of this cDNA, similar to that of the transcript shown by RNA gel blotting, suggested that it encoded a full-length or nearly full-length cDNA (Figure 1). Two ATG codons at base pairs 124 and 178, with surrounding sequences that reasonably conformed to the consensus initiation sequence described for dicotyledonous plants (Cavener and Ray, 1991), were identified at the beginning of the longest open reading frame of the cDNA. If the translation initiation were assigned at the most upstream position, this would yield a cDNA with a 123-bp 5' untranslated region followed by a 2340-bp open reading frame encoding a polypeptide of 780 amino acids with a calculated molecular mass of 84.7 kD. No potential polyadenylation signal was found in the 147-bp 3' untranslated region. Analysis of the GenBank data base revealed the present tobacco cDNA to be a novel member of the CIC family. For this reason and the functional arguments developed below, this cDNA was named CIC-Ntl (for CIC

Table 1. Binary Comparison of the Amino Acid Sequences of Tobacco CIC-Ntl, Rat CIC-1, Rat CIC-2, Human CIC-N4, and Yeast GEF1

<table>
<thead>
<tr>
<th>CIC-Ntl</th>
<th>CIC-1a</th>
<th>CIC-2a</th>
<th>CIC-N4d</th>
<th>GEF1e</th>
</tr>
</thead>
<tbody>
<tr>
<td>GEF1a</td>
<td>24 (50)</td>
<td>26 (52)</td>
<td>32 (57)</td>
<td>27 (52)</td>
</tr>
<tr>
<td>CIC-Ntl</td>
<td>23 (47)</td>
<td>24 (50)</td>
<td>30 (55)</td>
<td></td>
</tr>
<tr>
<td>CIC-N4d</td>
<td>29 (54)</td>
<td>25 (51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIC-2c</td>
<td>53 (70)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The results were obtained using the Bestfit program of the Genetics Computer Group software package (Devereux et al., 1984). Gap and length weight were fixed to 5.00 and 0.30, respectively. Values that are not within parentheses represent percentage of identity. Numbers within parentheses indicate percentage of similarity. Of all CIC members reported in the GenBank data base, CIC-1 and CIC-N4 represent the farthest and the closest homologs of CIC-Ntl, respectively.

b Steinmayer et al. (1992).
c Thiemann et al. (1992).
d Thiemann et al. (1992).
e Thiemann et al. (1992).
Figure 2. Comparison of CIC-Ntl with Its Animal and Yeast CIC Homologs.

(A) Amino acid sequence alignment of CIC-Ntl with T. marmorata CIC-0 (Jentsch et al., 1990); CIC-1 (Steinmeyer et al., 1991), CIC-2 (Thiemann et al., 1992), CIC-3 (Kawasaki et al., 1994), CIC-K1 (Uchida et al., 1993), and CIC-K2 (Adachi et al., 1994) from rat; human CIC-N4 (van Slegtenhorst et al., 1994); and yeast GEFl (Greene et al., 1993) in the most conserved region. Alignment was obtained using the Pileup and Prettybox programs of the Genetics Computer Group software package (Devereux et al., 1984). Dots indicate gaps to maximize alignment. Identical amino acid residues are shaded in black. Similar amino acid residues are shaded in gray. PCR primers used for amplification are underlined.

(B) Hydrophobicity analysis of the deduced amino acid sequence of CIC-Ntl and CIC-1. The mean hydrophobicity index was computed, according to the algorithm of Kyte and Doolittle (1982), with a window of 11 residues.
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A -30 mV

B -150 mV

C 1 µA

Figure 3. CIC-Ntl-Induced Currents in Xenopus Oocytes.

(A) and (B) Representative two-electrode voltage clamp traces from oocytes previously injected with CIC-Ntl antisense (A) or sense (B) cRNAs. The voltage clamp step protocol as shown in the inset was as follows: voltage was stepped for 30 sec from a holding potential of -30 mV to values between +60 and -150 mV in -10-mV increments. Interpulse duration at -30 mV was 2 min. Current traces recorded at -150 mV can be fitted by a simple exponential with a time constant of 8.1 ± 0.9 sec (n = 6). Note the slow deactivation of the currents at the end of the voltage step on return to -30 mV.

(C) Current-voltage relationship for uninjected oocytes (○; n = 3) and for oocytes injected with CIC-Ntl antisense (□; n = 3) or sense (●; n = 6) cRNAs (mean ± se). Currents were measured after 25 sec at the indicated potential. All recordings were done in OR2 medium supplemented with 1 mM CaCl2. No leakage subtraction protocol was used.

CIC-Ntl Currents Are Carried by Anions

Inward currents may correspond to cation influx or anion efflux across the oocyte membrane. Replacement in the oocyte bathing medium of sodium ions, the main extracellular cation, by presumably impermeant ions, such as cesium (Figures 4A and 4B) or Tris (data not shown), did not affect the CIC-Ntl inward currents, suggesting that cation influx cannot account for the observed currents. Replacement of extracellular chloride by sulfate or nitrate did not alter the amplitude of the inward currents activated upon hyperpolarization. However, chloride substitution by sulfate, but not by nitrate, strongly reduced the outward tail currents observed on subsequent depolarization (Figure 4C). These results suggested that the CIC-Ntl currents were efficiently carried by anions, such as Cl⁻ and NO₃⁻, but less so by SO₄²⁻. Figure 5 shows a quantitative study of outward tail current amplitudes in the presence of various extracellular anions. These analyses indicated that in addition to Cl⁻ and NO₃⁻, CIC-Ntl channels were highly permeable to I⁻ and Br⁻ but much less so to malate, sulfate, and glutamate.

To determine further the ionic selectivity of the CIC-Ntl currents, we examined the effects of external chloride concentration ([Cl⁻]) on the reversal potential of the tail currents. Figure 6 shows a semilogarithmic plot of this reversal potential as a function of [Cl⁻]. When external chloride was replaced...
Figure 5. Effects of Extracellular Anions on Outward Tail Current Amplitudes.

Oocytes were hyperpolarized at -150 mV for 30 sec to activate CIC-Ntl currents and subsequently clamped at +20 mV for 10 sec as described in Figure 4A. Time-dependent activation and tail currents were measured for oocytes successively bathed in OR2 supplemented with 1 mM CaCl2 (Cl) and in similar media in which NaCl was replaced by NaBr (Br), NaI (I), NaN03 (NO3), sodium-malate (Mal), Na2SO4 (SO4), or sodium-glutamate (Glu). For each individual measurement, the outward tail current amplitude was normalized to the amplitude of the corresponding activation current. The mean ± SE tail current amplitude determined for each extracellular anion was expressed as the percentage of the value measured in the presence of extracellular chloride (TailCl). Numbers within parentheses represent the number of oocytes tested under each condition.

by sulfate, the reversal potential became more positive, as expected for a chloride-permeable current. The reversal potential increased by 16 mV for a 10-fold decrease of [Cl]o from 80 to 8 mM, although the Nernst equation for a perfectly selective chloride current predicts a 58.5-mV change for a 10-fold change in the concentration gradient. However, for [Cl]o > 80 mM, the experimental data apparently converged toward the Nernst plot of an ideal chloride channel. Together, these data indicate that the inward currents elicited by CIC-Ntl were not perfectly selective for chloride over sulfate. In contrast, they showed a strong selectivity for chloride over sodium. We conclude that the CIC-Ntl currents activated by hyperpolarization of the oocyte membrane are anionic. The inward currents observed in these conditions mostly reflect a chloride efflux.

CIC-Ntl Inward Chloride Currents Can Be Modulated by Extracellular Anions, Calcium, and Anion Channel Blockers

While we were characterizing the ionic selectivity of the CIC-Ntl currents, we observed that replacement of extracellular chloride by large-sized anions, such as Hepes (Figure 7), Mes, or malate (data not shown), reduced instead of increased the amplitude of the inward currents, as would be expected from an increasing driving force for chloride efflux. This effect followed at least in part from an increase in the time constant of current activation. Reduction in the amplitude of inward currents was not observed for chloride substitution by ions such as I-, Br-, NO3-, or SO42-. These results suggest that anions differentially interact at the outer face of the anion channel protein and interfere with channel activation and anion efflux at hyperpolarized membrane potentials.

A number of chloride currents in plant cells have been reported to be calcium dependent. Figure 8A shows that an increase in extracellular calcium led to an inhibition of the CIC-Ntl currents observed at hyperpolarized membrane potentials. The dose–response of such inhibition at -150 mV (Figure 8B) shows that half and complete inhibition were obtained in the range of 1 and 10 mM extracellular calcium, respectively. A similar calcium inhibition profile was obtained in oocytes that were intracellularly injected with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (Figure 8B), suggesting that the block of CIC-Ntl currents by Ca2+ occurred at the extracellular side of the membrane.

Specific channel blockers might help in characterizing CIC-Ntl function in plant membranes. Several known Cl– channel blockers were tested for activity on the CIC-Ntl–induced inward currents in oocytes. They were blocked by 1 mM 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulfonic acid (SITS) (-79.5 ± 16.1%; n = 4; Figure 9A), slightly inhibited by 1 mM

Figure 6. Effects of Extracellular Chloride on the Reversal Potential of Tail Currents.

After a 30-sec hyperpolarization step at -150 mV, the oocyte membrane potential was stepped to values between -30 and +10 mV in 5-mV increments. Reversal potential was determined in a series of OR2-derived media in which Na2SO4 was progressively substituted for NaCl. Reversal potential measured from tail currents was plotted as a function of extracellular chloride concentration (mean ± SE; n = 6). The dashed line is the Nernst relationship for a perfectly selective chloride current.
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Figure 7. Modulation of CIC-Ntl Currents by Extracellular Anions.
Oocytes were successively tested in different media in which NaCl was progressively replaced by sodium-Hepes with constant osmolarity. Time-dependent currents were measured after 30 sec at -150 mV and normalized for each oocyte to the amplitude of the currents in the medium with 100 mM chloride (mean ± SE; n = 5).

4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (-25 ± 17%; n = 4; Figure 9B), insensitive to 1 mM anthracene-9-carboxylic acid (+2 ± 8%; n = 4), and slightly stimulated by 1 mM niflumic acid (+26 ± 14%; n = 4; Figure 9C) and 0.1 mM 5-nitro-2-(3-phenylpropylamino)benzoic acid (+42 ± 8%; n = 4).

DISCUSSION

In the past few years, an increasing number of homologs have been described in the CIC family of membrane proteins (Jentsch et al., 1995). Taking advantage of the high conservation of discrete amino acid motifs among the CIC members, we isolated CIC-Ntl from tobacco. Sequence analysis revealed that CIC-Ntl displays a core domain and a hydropathy profile characteristic of the CIC family. The roles of these key structural features in CIC assembly and activity remain to be elucidated. CIC-Ntl cloning extends the CIC family to higher plants. The present work agrees with the reports of CIC homologs in yeast (Greene et al., 1993) and more recently in Escherichia coli (Fujita et al., 1994). Together, these results support the idea that CIC sequences appeared early in evolution and have been conserved after the divergence of plant and animal kingdoms.

Expression of CIC-Ntl in Xenopus oocytes was found to elicit large inward currents upon oocyte membrane hyperpolarization. The effects of extracellular ions on the amplitude and on the reversal potential of these currents demonstrate that they are carried mainly by anions. These results are consistent with the voltage-dependent chloride channel activity previously assigned to some of the animal CIC homologs (Jentsch et al., 1995). Distinct current profiles have been obtained after oocyte expression of different CIC members, such as CIC-0, CIC-1, CIC-2, and CIC-K1 (Jentsch et al., 1995). CIC-Ntl–elicited current behavior in oocytes shares striking similarities with those expressed by rat CIC-2 (Thiemann et al., 1992), especially in their kinetics, voltage dependency, and poor sensitivity to classical Cl⁻ channel blockers. However, rat CIC-2 has been described as being activated by hypotonicity (Gründer et al., 1992); such a feature was not found in CIC-Ntl (data not shown).

Figure 8. Block of CIC-Ntl Currents by Extracellular Calcium.
(A) Current–voltage relationship of a representative oocyte bathed in OR2 in the absence of calcium (O) or in the presence of 1 mM (●), 3 mM (●), or 10 mM (▲) CaCl₂. Time-dependent currents were measured after 15 sec at the indicated potential. (B) Effects of extracellular calcium concentration on membrane currents elicited by a 15-sec pulse at -150 mV. Individual oocytes were successively tested at all calcium concentrations indicated. Results were normalized for each oocyte to the amplitude of the currents in the medium without calcium (mean ± SE). (O), CIC-Ntl–injected oocytes (n = 7); (●), CIC-Ntl–injected oocytes 20 to 75 min after an injection of 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (n = 6). We found that an injection of BAPTA decreases the rate of current deactivation on return to the holding potential of -30 mV. For this reason, for a given condition, we considered only the time-dependent currents activated during the corresponding pulse.
terization in oocytes, compelling evidence has accumulated showing that these proteins are primarily membrane channels and not channel regulators. For instance, the electrophysiological characteristics of CIC-3 established by oocyte expression were confirmed in Chinese hamster ovary–transfected cells (Kawasaki et al., 1995). The currents induced in oocytes by CIC-1 and CIC-K1 are fully consistent with previous transport measurements in the organs expressing these channels, the skeletal muscle (Steinmeyer et al., 1991) and the kidney ascending limb (Uchida et al., 1995), respectively. Finally, the single-channel activities observed in the electric organ of T. marmorata, in oocytes expressing CIC-O (Bauer et al., 1991), and in liposomes containing purified CIC-O (Middleton et al., 1994) display the same characteristics. In particular, similar “double-barreled” gating kinetics could be observed in each case. In conclusion, the homology of CIC-Nt1 to animal CICs, together with the finding that its functional expression in oocytes elicits large chloride currents, leads us to propose that tobacco CIC-Nt1 encodes a voltage-dependent anion channel.

Expression of CIC-Nt1 elicited large inward chloride currents in the Xenopus oocyte membrane. Yet, the expression of ion channels in these cells cannot be taken as unambiguous evidence for the protein expressed indeed forming a membrane channel. The CIC-Nt1 currents we obtained had features similar to those of an endogenous hyperpolarization-activated chloride current, that is, slow activation, block by extracellular calcium, sensitivity to SITS, and insensitivity to other classical chloride channel inhibitors (C. Lurin, D. Geelen, H. Barbier-Brygoo, J. Guern, and C. Maurel, unpublished results; Kowdley et al., 1994; Shimbo et al., 1995). However, the voltage activation thresholds of the endogenous and the CIC-Nt1–induced currents are clearly different. The endogenous current requires extreme hyperpolarization to at least −180 mV for activation, whereas CIC-Nt1–induced currents activate at potentials negative to −120 mV. On the other hand, Shimbo et al. (1995) have recently investigated the functional expression of various membrane proteins, such as IsK, phospholemman, and two influenza virus proteins, in oocytes. All of these proteins have been shown to have intrinsic channel activity. However, Shimbo et al. (1995) suggested that in each case, the currents observed after protein expression in oocytes followed in part from the modification of the endogenous chloride channels shifting their activation thresholds toward −120 mV. At present, we cannot totally exclude the possibility that functional expression of CIC-Nt1 in oocytes may also produce similar effects.

The currents induced by CIC-Nt1 in oocytes show regulatory features that have relevance to plant cell physiology. The apparent range of CIC-Nt1 voltage activation in oocytes is closer to the potential of the plant plasma membrane than to that of the tonoplast. However, CIC-Nt1 might correspond to either one of the anion channels that have been described as being activated upon hyperpolarization of the plasma membrane or the tonoplast (Hedrich and Becker, 1994). The slow activation and the lack of inactivation of CIC-Nt1 currents in oocytes are reminiscent of some of the channels thought to assure sustained trans-membrane anionic fluxes (Schauf and Wilson, 1987; Cerana et al., 1995). In conditions in which the H+ pumps are highly active, these channels may provide a mean for sustained membrane depolarization, long-term nutrient transport, and/or cell turgor regulation. In addition to their voltage dependence, the CIC-Nt1 currents can be modulated by extracytoplasmic anions, as was also reported for anion channels in the plant plasma membrane or tonoplast (Hedrich and Marten, 1993; Plant et al., 1994). Finally, regulation of CIC-Nt1 activity by extracytoplasmic calcium in the millimolar range is consistent with the calcium concentration reported in the vacuole as well as in the cell wall.

Whereas CIC-Nt1 channel characteristics and its localization in plant cells still need to be determined, our work suggests that CIC-Nt1 cDNA encodes a voltage-dependent anion channel. This provides a unique molecular probe for the study of chloride channels in plants.

FIGURE 9. Effects of Chloride Channel Blockers on CIC-Nt1–Induced Currents.

Currents were elicited by a 30-sec hyperpolarizing pulse to −150 mV. Individual current traces were recorded for representative oocytes before (○) and after a 10-min incubation in the presence of the following Cl− channel blockers.

(A) 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS; 1 mM).
(B) 4,4′-Disothiocyanostilbene-2,2′-disulfonic acid (DIDS; 1 mM).
(C) Niflumic acid (NA; 1 mM).

METHODS

Reverse Transcript–Polymerase Chain Reaction, cDNA Cloning, and Sequencing

Degenerate oligonucleotide primers were designed corresponding to two amino acid motifs (GKEGPxyVH and PxGGVLF) highly conserved in the animal and yeast voltage-dependent chloride channel (CIC) homologs. Degeneracy of primers was restricted by considering plant codon bias for translation (Murray et al., 1989). The primers that allowed successful amplification are as follows: sense strand, 5′-GG(A/T)AA-(A/G)GA(A/G)GGNCCNATGGTNCM'; antisense strand, 5′-AA(G/A)AG-(A/C)AC(A/T)CCNCCNACNGG-3′. One microgram of poly(A)+ RNA from Nicotiana tabacum cv Xanthi suspension-cultured cells was reverse transcribed using avian myeloblastosis virus (Promega, Madison,
at 94°C, cycles of polymerase chain reaction (PCR), each cycle comprising 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C). Reactions were performed in 50 μL of mixtures containing 50 pmol of each primer, 0.2 mM dideoxynucleotides, and 0.5 μL of Taq polymerase (Applied, Illkirch, France) in the corresponding buffer. Amplified products were cloned in T-tailed pBluescript KS+ (Stratagene, La Jolla, CA) and sequenced. A 197-bp product (NtI) that represented a putative plant CIC homolog was selected for further characterization.

Approximately 105 clones of a tobacco cultivar Xanthi plantlet cDNA library constructed in λZAPII (Stratagene) (kindly provided by A. Dargeviculite, CNRS, Gif sur Yvette) were screened at high stringency using standard procedures (Sambrook et al., 1989), with the NtI fragment labeled with α-32P-dCTP. Hybridizations were done in 5 x SSC in 0.15 M NaCl, 0.015 M sodium citrate), 7% SDS, and in the corresponding buffer. Amplified products were cloned in T-tailed pBluescript SK− (Stratagene, La Jolla, CA) and sequenced. Plasmid pCIC-NtI carrying an EcoRI-EcoRI 2610-bp insert was selected for further analysis.

Both strands of the pCIC-NtI insert were fully sequenced by the chain termination method (Sanger et al., 1977), using a T7 sequencing kit (Pharmacia, Uppsala, Sweden). For this, a set of overlapping deletions created in the sense direction by exonuclease I digestion and in the antisense direction by restriction enzyme digestion were used. Sequence data were analyzed using the Genetics Computer Group (Madison, WI) Version 8.0 software package (Devereux et al., 1984).

**Oocyte Expression**

The 2610-bp cDNA fragment encoding CIC-Nt1 was cloned into the EcoRI site of T7TS, a pGEM4Z-derived vector containing the 5' and 3' untranslated regions of a Xenopus β-globin cDNA (Abrami et al., 1994). Capped complementary RNA (cRNA) was synthesized in vitro using T7 RNA polymerase (Promega), as previously described (Maurel et al., 1993), and yielded, according to the cDNA orientation in the T7TS vector, a sense cRNA encoding the CIC-NtI protein or its antisense counterpart. CIC-NtI antisense cRNA was used as a negative control in oocyte expression experiments.

Stage V and VI Xenopus oocytes were prepared as described previously (Maurel et al., 1993). They were injected with 50 nL of in vitro-transcribed cRNA (0.3 to 2 μM resistance) and maintained under voltage clamp with a GeneClamp 500 two-electrode amplifier (Axon Instruments, Foster City, CA). Bath clamp was achieved by a two-electrode virtual ground circuit with Axoclamp 2B (Axon Instruments). Voltage pulses were made from a holding potential of ~30 mV. Standard membrane current measurements were performed in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM Hepes, pH 7.5) supplemented with 1 mM CaCl2.

The effects of extracellular anions on the currents were tested in media containing 2 mM KCl, 2 mM MgCl2, 1 mM CaCl2, 5 mM Hepes, pH 7.5, plus NaCl and/or sodium-Hepes, pH 7.5, with a constant osmolality of 220 mosmol/kg. The Cl− concentrations used were 8, 28, 58, 108, and 128 mM.

The effects of extracellular calcium on the hyperpolarization-induced membrane currents were characterized in OR2 media supplemented with MgCl2 and CaCl2, with MgCl2 plus CaCl2 totaling 11 mM. Membrane currents were characterized on individual oocytes sequentially superfused with media of increasing or decreasing calcium concentrations. When indicated, oocytes were injected with 50 nL of 50 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (the estimated final concentration in the whole oocyte was 2.5 mM) and incubated in Barth's solution for 20 to 75 min before assaying the effects of extracellular calcium on the membrane currents. The Cl− channel blockers niflumic acid, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), and anthranec-9-carboxylic acid were used at a concentration of 1 mM and 5-nitro-2-(3-phenylpropylamino)benzoic acid at a concentration of 0.1 mM in OR2 supplemented with 1 mM CaCl2. The percentage of inhibition was calculated by measuring the time-dependent currents activated during a 30-sec pulse to −150 mV before and after a 10-min incubation in the presence of the blocker.

**REFERENCES**

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**Two-Electrode Voltage Clamp**

Individual oocytes were impaled in a perfused bath at room temperature with microelectrodes filled with 1 M KCl (0.8 to 2 MΩ resistance) and maintained under voltage clamp with a GeneClamp 500 two-electrode amplifier (Axon Instruments, Foster City, CA). Bath clamp was achieved by a two-electrode virtual ground circuit with Axoclamp 2B (Axon Instruments). Voltage pulse protocols and data acquisition were performed by a pClamp program (Axon Instruments). All measurements were performed in OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM Hepes, pH 7.5) supplemented with 1 mM CaCl2.


