Expression of Human pICln and CIC-6 in Xenopus Oocytes Induces an Identical Endogenous Chloride Conductance*

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pICln is a protein that induces an outwardly rectifying, nucleotide-sensitive chloride current (I\textsubscript{Cl}) when expressed in Xenopus oocytes, but its precise function (plasma-membrane anion channel versus cytosolic regulator of a channel) remains controversial. We now report that a chloride current identical to \textit{I}_{\textit{Cl}} is induced when Xenopus oocytes are injected with human CIC-6 RNA. Indeed, both the pICln and the CIC-6 induced currents are outwardly rectifying, they inactivate slowly at positive potentials and have an anion permeability sequence NO\textsubscript{3} \textsuperscript{>} > I\textsuperscript{>} > Br\textsuperscript{>} > Cl\textsuperscript{>} > gluconate. Cyclamate, NPPB, and extracellular cAMP block the induced currents. The success rate of current expression is significantly increased when the injected Xenopus oocytes are incubated at a higher temperature (24 or 37 °C) prior to the analysis. In addition, the \textit{I}_{\textit{Cl}} current was detected in 6.2% of noninjected control Xenopus oocytes. We therefore conclude that the \textit{I}_{\textit{Cl}} current in Xenopus oocytes corresponds to an endogenous conductance that can be activated by expression of structurally unrelated proteins. Furthermore, functional, biochemical, and morphological observations did not support the notion that pICln resides in the plasma membrane either permanently or transiently after cell swelling. Thus, it is unlikely that pICln forms the channel that is responsible for the \textit{I}_{\textit{Cl}} current in Xenopus oocytes.

Irrespective of the relation between pICln and \textit{I}_{\textit{Cl, swell}}, one major unresolved problem is how expression of mammalian pICln in Xenopus oocytes generates a specific Cl\textsuperscript{-} current. This was originally explained by assuming that pICln was a plasma membrane-spanning protein that constituted the anion channel itself (4). However, Krapivinsky et al. (2) showed that the majority of pICln resided in the cytosol, and they concluded that pICln was a cytosolic regulator of an endogenous, plasma membrane–located anion channel. Recently, it has been suggested that pICln, in spite of its cytosolic location, can still function as a plasma membrane–located anion channel if one assumes a hirnomaal distribution for pICln with a subdivision residing in the plasma membrane (6, 7). Moreover, the ratio between cytosolic and plasma membrane pICln could vary, depending on specific stimuli such as cell swelling (10).

To further characterize the \textit{I}_{\textit{Cl}} chloride current and to address the role of pICln, we have expressed human pICln in Xenopus oocytes. First of all, we observed that the success rate of \textit{I}_{\textit{Cl}} expression in Xenopus oocytes injected with pICln RNA markedly depended on the incubation temperature of the oocytes. Furthermore, the \textit{I}_{\textit{Cl}} current was also triggered in Xenopus oocytes injected with RNA coding for human CIC-6, a protein that is structurally unrelated to pICln. Finally, we also argue against a plasma membrane location for pICln.

**EXPERIMENTAL PROCEDURES**

PCR, reverse transcription-PCR, and Vector Construction—A human cdNA clone for pICln (accession number X91788; see Ref. 1) was PCR-mutagenized by replacing the 5’-untranslated region with an EcoRI/HindIII/BamHI linker. This allowed subcloning of human pICln cdNA as a BamHI fragment in the RNA transcription vector pGEMHE (11), yielding the pGEMHE/EHBhIClnORF vector. The human CIC-6 clone was constructed as follows. First we amplified by reverse transcription-PCR the 5’-end of human CIC-6 (nucleotides 202–1229 of the published open reading frame; see Ref. 12). Reverse transcription of 1 μg of total RNA of human K562 cells (ATCC CCL 243) was performed as described (13) except that random primers were used instead of oligo(dT) primers. The PCR reaction was carried out with Pfu polymerase (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The PCR fragment was then digested with BamHI (in forward primer) and XbaI (internal site at nucleotide 792). The remainder of the CIC-6 open reading frame (nucleotides 793–2610) and part of the 3’-untranslated region were isolated as an XbaI-HindIII fragment from the HA0519 clone. This is a partial human CIC-6 cdNA clone isolated from a human myeloid cell line by Miyajima and co-workers (EMBL/GenBank accession number D28475). The BamHI-XbaI PCR fragment and the XbaI-HindIII fragment were ligated in a pBluescript vector digested with BamHI-HindIII. Nucleotides 1–201 of the open reading frame were then amplified by reverse transcription-PCR from human K562 RNA and inserted upstream of the CIC-6 sequence via a NcoI site. This created a pBluescript vector containing the complete CIC-6 open

*The abbreviations used are: PCR, polymerase chain reaction; PBS, phosphate-buffered saline; NPPB, 5-nitro-2-(3-phenylpropylaminobenzoic acid.

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Expression of Human pICln and CIC-6 in Xenopus Oocytes and Electrophysiology—The pGEMHE/CIC-6 vector was cut with SpH1, and the pGEMHE/EHBDIClchOIF vector was cut with HindIII. The linearized DNA was purified (QIAquick DNA purification protocol) and transcribed using T7 RNA polymerase (RiboMAX System, Promega). RNAs were extracted with phenol/chloroform and ethanol-precipitated. Stage V–VI Xenopus oocytes were isolated by partial ovariectomy and defolliculated by collagenase treatment. Between 2 and 4 h after defolliculation, oocytes were injected with 50 nl of 1–100 ng of purified pICln or CIC-6 RNA. The oocytes were then incubated at 18 °C for 2–4 days in ND-96 solution supplemented with gentamicin sulfate (50 mg/ml). ND-96 contains 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, pH 7.5.

Whole-cell currents from oocytes were recorded using the two-micro-electrode voltage clamp technique. Resistances of voltage and current electrodes filled with 3 M KCl were 0.5–2 MΩ. Current was sampled at 500- or 2000-μs intervals and filtered at 1 or 0.1 kHz, respectively, using a quadrupole low pass Bessel filter. To eliminate the effect of voltage drop across the bath-grounding electrode, the bath potential was actively controlled. Linear components of capacity and leak currents were not subtracted. The bath solution was ND-96 except for the study of the anion permeability sequence, where NaCl (96 mM) was replaced with various sodium anions (96 mM). These experiments were performed using an agar bridge. Three voltage protocols were used during the analysis: (i) in step protocol 1, from a holding potential of −20 mV, oocytes were clamped for 800 ms at −100 mV to +100 mV spaced 20 mV; (ii) in step protocol 2, oocytes were held at −70 mV, and 800-ms pulses to −40, 0, or +40 mV were applied; and (iii) in the linear voltage ramp protocol, oocytes were held at −20 mV, and a linear voltage ramp from −100 mV to +100 mV (0.4 V/s) was applied. Electrophysiological experiments were performed at room temperature on Xenopus oocytes that been permanently incubated at 18 °C or that had been subjected to a temperature elevation (24 °C for 3 h or 37 °C for 30 min) prior to the electrophysiological analysis.

Permeability ratios (PNO3/PCl) for various anions were calculated using the formula, \( P_{NO3} / P_{Cl} = (Cl− \times exp(\Delta G_o / RT)) / Cl_{ext}/IX_{\infty} \), with ΔG being the shift in reversal potential, \( Cl \) the extracellular Cl− concentration in ND-96, \( X\) the extracellular anion concentration in anion-substituted ND-96, and \( Cl_{ext} \) the remaining Cl− concentration in the anion-substituted media. Numerical data are represented as mean ± S.E.

Expression of human pICln in Xenopus oocytes was verified by Western blot analysis using a polyclonal anti-pICln antiserum (1). Noninjected and pICln RNA-injected oocytes were lysed in a hypotonic buffer containing 25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2.5 mM EGTA. The lysate was centrifuged, and the supernatant was stored at −20 °C. 50 μg of Xenopus protein extract was analyzed on Western blot as described previously (1).

Preparation of Cytosolic and Microsomal Protein Fractions and Immunoblotting—Subcellular fractionation of a human endothelial cell line (EA.hy926 cells; see Ref. 14) was performed by step centrifugation. Cultured cells were washed with phosphate-buffered saline (PBS) containing 1 mM EDTA, trypsinized, pelleted, and resuspended in 1 ml of isotonic PBS or 1 ml of hypotonic PBS (0.6 ml of PBS, 0.4 ml of H2O). After a 5-min incubation at room temperature or at 37 °C, cells were lysed by sonication. Nuclei plus mitochondria were sedimented by centrifugation at 10000 x g. The supernatant was then fractionated into cytosolic and microsomal fractions by ultracentrifugation at 100000 x g for 30 min. The microsomal fraction was resuspended in 20 mM Tris-HCl, pH 7.4, supplemented with 300 mM sucrose.

Cytosolic (50-μg) and microsomal (200-μg) protein fractions were separated on SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidene fluoride membranes by semidy electrollocttration. Human pICln was detected with a polyclonal anti-pICln antiserum (1:500 dilution; see Ref. 1) using the Amersham ECL method. Secondary antibodies were peroxidase-labeled swine anti-rabbit immunoglobulins (DAKO A/S) diluted 1:1000.

Confocal Immunofluorescence Study of pICln Localization in EA.hy926 Cells—Anti-pICln antibodies were first affinity-purified from a polyclonal anti-pICln antiserum using a hexahistidine-tagged human pICln protein, which was bound to a polyvinylidene fluoride micropermeable membrane strip (Immobilon; Millipore). EA.hy926 endothelial cells were grown on gelatin-coated chamber slides (Lab Tek; Nunc Inc.). Prior to fixation in PBS containing 3% paraformaldehyde, cells were incubated for 5 min with isotonic or hypotonic (40% reduction in tonicity) PBS. Cells were permeabлизed with 0.5% Triton X-100 and blocked with 10% goat serum in PBS for 1 h. They were then incubated with the affinity-purified polyclonal anti-pICln antibodies for 36 h at 4 °C and thereafter with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Sigma) at room temperature for 1 h. A control, cells incubated only with the secondary antibodies were analyzed in parallel. Cells were imaged using a Bio-Rad MRC 1000 confocal microscope with a ×400 objective lens, a 0.7 NA. The images were averaged from 5 and 10 frames. Both confocal (iris confocal aperture, 2 mm), and nonconfocal images (768 x 512 lines) were stored.

Cell Volume Measurements—EA.hy926 cells were grown on glass coverslips, and cell height was monitored as described by Van Driessche et al. (15).

RESULTS

Expression of the pICln-associated Chloride Current in Xenopus Oocytes Is Temperature-dependent—It has previously been reported that mammalian human pICln in Xenopus oocytes resulted in an outwardly rectifying chloride current (Icl) that slowly inactivated at positive potentials and that could be blocked by extracellular nucleotides (4, 16, 17). However, our initial attempts to functionally express human pICln in Xenopus oocytes were rather unsuccessful; less than 10% of Xenopus oocytes injected with human pICln RNA displayed currents with an Icl phenotype. The failure to functionally express Icl could not be ascribed to the defolliculation method, since both collagenase-defolliculated and manually defollicolated Xenopus oocytes gave similar negative results (data not shown). We then accidently discovered that functional expression in some oocyte batches was strongly promoted by incubating the oocytes at a higher temperature (24 °C for 3 h or 37 °C for 30 min) prior to the electrophysiological analysis at room temperature (Fig. 1A). Fig. 1B compares the current amplitude at +100 mV of injected and noninjected Xenopus oocytes when they were kept at 18 °C and when the incubation temperature was raised to 24 °C for at least 3 h prior to the electrophysiological analysis at room temperature (n = 6 for each experimental condition; all oocytes derived from a single batch). In this experiment, only Xenopus oocytes that had been injected with human pICln and that had been subjected to a temperature shift displayed Icl-type currents (see Fig. 2, A, C, and E, for a characterization of the current). From then on, we routinely included a minimum incubation of 3 h at 24 °C in our experimental protocol.

The absence of Icl-type currents in injected Xenopus oocytes permanently incubated at 18 °C could not be explained by deficient translation of human pICln RNA. Western blot analysis of oocyte extracts confirmed the presence of human pICln protein in Xenopus oocytes that had been injected with human pICln RNA and that had been kept at 18 °C (see Fig. 1C). The overall quality of the Xenopus oocytes within one batch and among different batches was always checked in parallel experiments in which we injected Xenopus oocytes with RNA coding for a Kv1.1 voltage-gated potassium channel (RCR1) (18). Typical Icl currents were elicited in all of the injected oocytes independent of the incubation temperature (data not shown).

Human pICln and CIC-6 Induce Identical Chloride Currents when Expressed in Xenopus Oocytes—The kinetics, the anion permeability, and the pharmacology of Icl currents induced by heterologous expression of mammalian pICln in Xenopus oocytes have been previously characterized (4, 9). Icl features are identical in manually defollicolated (4) and collagenase-defollicolated (9) Xenopus oocytes, and they are summarized in Fig. 2, A, C, and E. Briefly, Icl is an outwardly rectifying current that slowly inactivates at positive membrane potentials (at least +60 mV). It is an anion-selective current with a permeability sequence NO3− > I− > Br− > Cl− > gluconate. The permeability ratios \( P_{NO3}/P_{Cl} \) calculated from shifts in reversal potential in anion-substituted media are as follows (n = 5): 1.35 ± 0.04 (NO3−), 1.19 ± 0.02 (I−), 1.07 ± 0.02 (Br−), and
Cyclamate acts as a channel blocker. IClin is blocked by NPPB (83 ± 6% block with 100 μM at 180 mV; n = 5) and extracellular cAMP. The cAMP block is clearly voltage-dependent, since it only affects the outward current: 5 mM cAMP blocks 42 ± 7% of the current at 180 mV versus 3.1 ± 4.3% at -80 mV (p < 0.5).

ClC-6 is a recently described member of the ClC chloride channel family (12). The functional characteristics and the physiological role of ClC-6 are still unknown, since functional expression of ClC-6 in Xenopus oocytes was reported to be negative (12). When we injected Xenopus oocytes with human ClC-6 RNA, we initially also obtained negative results. However, when we preincubated the Xenopus oocytes at a higher temperature prior to the analysis, we observed an outwardly rectifying current that inactivated slowly at positive potentials.
(at least +60 mV; see Fig. 2, B, D, and F, for a description of the current). For example, in one batch of *Xenopus* oocytes, all oocytes (either injected with CIC-6 RNA or H₂O-injected; n ≥ 10 for each condition) remained negative when they were continuously kept at 18 °C. In contrast, 10 of 14 oocytes injected with CIC-6 RNA acquired an I_Cln phenotype after a temperature shock (either >3 h at 24 °C or 30 min at 37 °C; data not shown). In this experiment, 1 of 14 H₂O-injected control *Xenopus* oocytes also became positive after temperature elevation.

In view of the similarities between the CIC-6-induced current and I_Cln, we then went on to compare in greater detail these two currents in *Xenopus* oocytes (Fig. 2). The CIC-6-induced currents were outwardly-rectifying, and they inactivated slowly at positive potentials. We quantified the rectification by calculating the ratio of the current amplitude at +55 mV to that at −95 mV. These potentials were chosen, since they are approximately equidistant from the Cl⁻ equilibrium potential in *Xenopus* oocytes. In ND-96 the rectification score (I_{+55mV}/I_{-95mV}) of the CIC-6-induced current was 6.9 ± 1.5 (n = 20), which is comparable with the 6.2 ± 0.8 score of the pICln-induced current. The CIC-6-induced currents reversed between −25 and −35 mV in ND-96, and they depended on the presence of extracellular anions, since substitution of extracellular NaCl with various sodium anion solutions changed the current amplitude and shifted the reversal potential of the induced current. Based on shifts in the reversal potential we obtained the following permeability sequence: NO₃⁻ > Br⁻ > Cl⁻ > gluconate. P_Na/P_Cl ratios calculated from shifts in reversal potential were as follows (n = 5): 1.37 ± 0.04 (NO₃⁻), 1.24 ± 0.03 (Br⁻), 1.12 ± 0.02 (Br⁻), and 0.46 ± 0.03 (gluconate). The CIC-6-induced current was blocked by cyclamate. Furthermore, NPPB blocked the CIC-6-induced current to a similar degree as the pICln-induced current (81 ± 5% block with 100 μM at +80 mV, n = 5). Extracellular cAMP only inhibited the outward current (45 ± 8% block with 5 mM cAMP at +80 mV versus 3.4 ± 1.8% block at −80 mV; n = 5). Thus, expression of two structurally nonrelated proteins in *Xenopus* oocytes induced a chloride current with identical biophysical and pharmacological characteristics.

**I_Cln Is Also Present in Noninjected or H₂O-injected Xenopus Oocytes**—A possible explanation for the observation that pICln and CIC-6 induce an identical Cl⁻ current in *Xenopus* oocytes is that these proteins activate, directly or indirectly, a Cl⁻ conductance that is endogenously present in *Xenopus* oocytes (see “Discussion”). One observation in favor of this interpretation is that an I_Cln-type current is occasionally observed in noninjected or H₂O-injected *Xenopus* oocytes. In a survey of 81 noninjected or H₂O-injected *Xenopus* oocytes, we observed 5 oocytes (6.2%) an I_Cln phenotype (outward rectification; slow inactivation at potentials of at least +60 mV; reversal potential about −30 mV). Similarly, Paulmichl et al. (4) reported that 36 of 943 H₂O-injected oocytes (3.8%) displayed an I_Cln phenotype.

**Control Xenopus Oocytes Contain a Conductance That Is Phenotypically Identical to the Nucleotide-resistant Mutant I_Cln**—Expression of mutated pICln proteins in *Xenopus* oocytes suggested a close link between pICln structure and I_Cln phenotype, since mutations in a putatively extracellular, glycine-rich region led to mutant currents that could no longer be blocked by extracellular nucleotides such as cAMP (4). However, these mutations also changed the kinetics and the Ca²⁺ dependence: in contrast to the wild type I_Cln, the mutant current activated slowly at positive potentials, and reducing extracellular Ca²⁺ decreased its amplitude. It was therefore concluded that pICln was a plasma membrane-spanning protein with an extracellular nucleotide binding site and extracellular Ca²⁺ binding sites. However, this interpretation should be treated cautiously, since the mutant phenotype (slow activation, dependence on extracellular Ca²⁺, tail currents) resembles a conductance that is endogenously present in *Xenopus* oocytes (e.g. the steady state current in Ref. 19). We demonstrated this by eliciting the “mutant phenotype” in noninjected *Xenopus* oocytes using the same test protocols as Paulmichl et al. (4). All *Xenopus* oocytes possessed a current that slowly activated during an 800-ms voltage step to +40 mV from a holding potential of −70 mV (Fig. 3). The time constant of activation at +40 mV was 105 ± 17 ms (n = 15), which is identical to the time constant reported by Paulmichl et al. (4) (100 ± 25 ms). The amplitude of this current at the end of an 800-ms pulse at +40 mV varied between 0.1 and 2.1 μA (0.62 ± 0.14 μA; n = 15). This current depended on extracellular Ca²⁺, since perfusion of the oocytes with ND-96 containing 1 mM EGTA reduced its amplitude to 60 ± 3% (n = 4). Furthermore, it was not sensitive to 5 mM extracellular cAMP (n = 5). This observation demonstrates that a current with a phenotype identical to the mutant I_Cln is also present in noninjected control *Xenopus* oocytes.

**Evidence against a Plasma Membrane Location of pICln**—Subcellular fractionation of mammalian cells has revealed that the majority of pICln resides in the cytosol and that a small part of pICln associates with the microsomal fraction (2, 10). In addition, it has been reported for NIH3T3 fibroblasts and LL-
CPK1 cells that a reduction of the extracellular osmolarity induces a translocation of pICln from the cytosolic to the microsomal fraction (10). Although we did not think that there is a direct link between pICln and I_{cl,swell} (9), we were still interested in specific conditions in which pICln could reside in the plasma membrane. We therefore studied the subcellular distribution of human pICln in a human endothelial cell line (EA.hy926) in which we had previously documented the expression of the pICln protein (1). Functional studies also indicated that EA.hy926 cells were sensitive to changes in extracellular osmolarity (data not shown). A reduction of the extracellular tonicity with 28% activated a volume-sensitive Cl\(^{-}\) current that was similar to I_{cl,swell} of other endothelial cell lines such as bovine pulmonary artery endothelial cells (CPAE, see Refs. 20 and 21) or human umbilical vein endothelial cells (22). With 140 mM Cs\(^{+}\) in the pipette solution and 5 mM Cs\(^{+}\) in the bath to block the inward K\(^{+}\) rectifier, I_{cl,swell} in EA.hy926 cells showed outward rectification, slow inactivation at positive potentials, and virtually no voltage dependence. Current densities at −80 mV in isotonic and hypotonic (28% reduction) conditions were, respectively, 2.8 ± 0.3 pA/picofarad (mean ± S.E.; n = 19) and 49.3 ± 5.2 pA/picofarad (mean ± S.E.; n = 19; p < 0.001). Using the method described by Van Driessche et al. (15) we directly tested whether EA.hy926 cells swell when the extracellular tonicity was lowered. A hypotonic stimulus (28 or 50% reduction) for 5 min induced an increase in cell height to, respectively, 138 ± 5% (mean ± S.E.; n = 36) or 163 ± 7% (mean ± S.E.; n = 29).

In view of the expression of pICln in EA.hy926 cells and in view of their sensitivity to changes in extracellular tonicity, we analyzed the subcellular distribution of pICln in these cells under isotonic and hypotonic conditions. Subcellular fractionation followed by Western blot analysis confirmed that the majority of pICln was recovered in the cytosolic fraction under isotonic conditions, indicating that pICln is a soluble and cytosolic protein (Fig. 4). A minor proportion of pICln was recovered in the microsomal fraction, indicating that the method was sufficiently sensitive to detect pICln in the membrane fraction under isotonic conditions. Importantly, the ratio between cytosolic and microsomal pICln did not change when the cells were subjected to a hypotonic stimulus (40% reduction in extracellular tonicity for 5 min; see Fig. 4). Identical results were obtained when the hypotonic treatment was performed at room temperature or at 37 °C.

The association with the microsomal fraction during subcellular fractionation does not necessarily mean that pICln is a plasma membrane protein. Indeed, the microsomal fraction is a mixed population consisting of plasma membrane and intracellular membrane vesicles. Moreover, the association can be merely peripheral, for example mediated by ionic interactions between pICln and membrane lipids or proteins. To address this problem, we studied the localization of pICln by immunofluorescence confocal microscopy in EA.hy926 cells. In this experiment, cells were incubated at room temperature for 5 min either with isotonic (control cells) or hypotonic (60% tonicity) PBS prior to fixation. Confocal sections showed the presence of pICln throughout the cytosol (Fig. 5). Importantly, the periphery of the cell was devoid of pICln. Furthermore, the intracellular distribution of pICln was unaffected when the cells were subjected to a hypotonic stimulus (Fig. 5). Confocal sections revealed an identical pattern of pICln distribution for control cells and cells subjected to a hypotonic challenge.

**DISCUSSION**

In this study we describe the induction of an identical Cl\(^{-}\) current by expression of two structurally unrelated proteins in *Xenopus* oocytes. The current induced by human pICln and ClC-6 corresponds phenotypically to the I_{cl} current observed after heterologous expression of other mammalian pICln proteins in *Xenopus* oocytes (4, 16, 17). In short, I_{cl} is an outwardly rectifying anion current with slow inactivation kinetics at positive potentials. Its permeability sequence is NO₃\(^{-}\) > I\(^{-}\) > Br\(^{-}\) > Cl\(^{-}\) > gluconate. It is blocked by cyclamate, NPPB, and extracellular cAMP. In *Xenopus* oocytes, the I_{cl} current can be clearly discriminated from the volume-activated chloride current (9). The success rate of I_{cl} expression was greatly enhanced in some *Xenopus* oocyte batches by incubating the *Xenopus* oocytes injected with pICln or ClC-6 RNA for ≥3 h at 24 °C or for 30 min at 37 °C prior to the electrophysiological analysis. Brandt et al. (12) mention that they incubated CIC-6 injected *Xenopus* oocytes at 18 °C for 2 or 3 days before current measurements. The absence of a temperature elevation may very well explain why they did not observe a chloride current in CIC-6-injected *Xenopus* oocytes. The temperature dependence was specific for I_{cl}, since functional expression of a voltage-dependent K\(^{+}\) channel (RCK1) did not require a preceding temperature shift. In principle, temperature-dependent processes can affect channel expression by interfering with the biosynthesis (translation, membrane insertion, and folding), the transport from rough endoplasmic reticulum to the plasma membrane, and/or the gating mechanism. As for pICln, we can rule out a temperature effect on translation of pICln RNAs, since we were able to detect human pICln in extracts of injected *Xenopus* oocytes that had only been incubated at 18 °C. A similar effect of incubation temperature on channel expression in *Xenopus* oocytes has been described for epithelial Na\(^{+}\) channels, in which case insertion into the plasma membrane seems to be the temperature-dependent step (23).

How can we explain the finding that pICln and ClC-6 induce an identical Cl\(^{-}\) current when expressed in *Xenopus* oocytes and that a similar current can be observed in a small minority of control *Xenopus* oocytes? There are, in principle, two alternative possibilities. (i) Both pICln and ClC-6 are anion channels.
pressed proteins, pUS oocytes (1, 2) and that the expression of a mutant pICln does not necessarily mean that pICln is an identical anion channel that is endogenously present in or a hypotonic buffer (40% reduction for 5 min; D). These conditions reveal more clearly the cytosolic localization of pICln, and importantly, they do not show evidence for a translocation of pICln to the plasma membrane after hypotonic stimulation. Parameters for laser microscopy were identical for panels A–B and C–D, respectively. The color scale (see vertical bar in B) ranges from blue (background signal) to white (saturating signal).

In principle, one could argue that the heterologously expressed proteins, i.e. human pICln and CIC-6, interact directly or indirectly with pICln that is endogenously present in Xenopus oocytes (1, 2) and that the Xenopus pICln forms the actual channel. This interpretation presupposes that pICln is an intrinsic membrane protein that spans the plasma membrane. However, in our opinion there are no compelling arguments in favor of this assertion. (i) The amino acid sequence of pICln contains no hydrophobic regions that are sufficiently long to traverse the membrane as an α-helix. A structural model has been proposed in which the transmembrane part of pICln consists of an amphipathic β-sheet (4). However, no experimental evidence in favor of this model has been presented. (ii) The changes in current phenotype (loss of block by extracellular nucleotides, sensitivity to extracellular Ca$^{2+}$) observed after expression of a mutant pICln do not necessarily mean that pICln spans the plasma membrane. As demonstrated, an identical phenotype was present in noninjected oocytes. (iii) The association of small amounts of pICln with the membrane fraction after cell fractionation does not necessarily imply that it is a transmembrane protein inserted into the plasma membrane. Alternative explanations are that pICln is a peripheral membrane protein and/or that it associates with intracellular membrane structures (endoplasmic reticulum, Golgi, endosomes, etc.) rather than with the plasma membrane. (iv) The preferential association with the soluble fraction (our data and Ref. 2) as well as the confocal immunofluorescence data point to a cytosolic location. (v) In contrast to Paulmichl et al. (10) we did not observe a shift of pICln to the plasma membrane upon reducing extracellular osmolarity. (vi) Krapivinsky et al. (2) have examined the distribution of endogenous pICln in Xenopus oocytes, and they were unable to identify pICln in oocyte microsomes. We therefore conclude that pICln is not a plasma membrane-located channel protein and, consequently, that the I$_{\text{Cln}}$ current is not carried by the pICln protein. As to CIC-6, it has formally not yet been proven that it resides in the plasma membrane. However, its structural relationship to well documented plasma membrane Cl$^{-}$ channels such as CIC-0 and CIC-1 and the presence of several hydrophobic segments in the hydrophathy analysis strongly suggest that CIC-6 is a membrane protein (12).

Our interpretation of the present data implies a molecular pathway that links a cytosolic protein, pICln, with the plasma membrane anion channel. Krapivinsky et al. (2) have shown that pICln forms oligomeric complexes with several cytosolic proteins, one of which has been identified as actin. In addition, a small amount of pICln was found to be associated with the membrane cytoskeleton (2). These observations are consistent with pICln forming part of a protein-protein interaction cascade that may be responsible for the activation of a plasma membrane anion channel. Clearly, more experimental work is required to establish the exact role of pICln in the regulation of membrane conductance in Xenopus oocytes.
quired to unravel this pathway. Similarly, it is not known how ClC-6 activates the endogenous channel. Since ClC-6 most likely resides in the membrane (see above), it may interact with the anion channel either directly or indirectly via one or more intervening proteins.

At present we do not know whether the activation of \( I_{\text{Cl}} \) is related to the proper physiological function of \( I_{\text{Cl}} \) and of ClC-6 or whether it represents a mere side effect of expressing exogenous proteins in Xenopus oocytes. Yet, the present data directly impinge on the functional models for \( I_{\text{Cl}} \) and its proposed physiological role as a volume-regulated anion channel and our previous observation that ClC-6 and \( I_{\text{Cl}} \) \( \text{swell} \) are two different currents (9) argue against a role for \( I_{\text{Cl}} \) as a volume-regulated anion channel or as a regulator thereof. As to the physiological role of ClC-6, there is as yet no formal evidence that it is a \( \text{Cl}^- \) channel, since expression studies have either yielded negative results (12) or the \( I_{\text{Cl}} \) phenotype (this study). The structural relationship of ClC-6 with proven \( \text{Cl}^- \) channels such as ClC-0, ClC-1, ClC-2, and ClC-5 is compatible with a role in \( \text{Cl}^- \) or anion transport, but further experiments are required to clarify this issue.

Finally, although Xenopus oocytes have proven to be a reliable and powerful tool to analyze ion channels, they may contain specific pitfalls when used to identify or characterize exogenous proteins. For example, at least four distinct endogenous \( \text{Cl}^- \) currents have been described in Xenopus oocytes: a \( \text{Ca}^{2+} \)-activated \( \text{Cl}^- \) current (30), a hyperpolarization-activated \( \text{Cl}^- \) current (31), a volume-activated \( \text{Cl}^- \) current (9, 32, 33), and the \( I_{\text{Cl}} \) current. Moreover, two of them can be activated by the expression of exogenous proteins. Expression of small integral membrane proteins such as phospholemman, MAT-8, IsK, SYN-C, and NB activates the hyperpolarization-activated \( \text{Cl}^- \) current (24–28), whereas \( I_{\text{Cl}} \) is induced by \( I_{\text{Cl}} \) and ClC-6.

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