Molecular Characterization of a Swelling-Induced Chloride Conductance Regulatory Protein, \( p_{Cl}\text{in} \)

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Summary

Cells maintain control of their volume by the passage of KCl and water across their membranes, but the regulatory proteins are unknown. Expression in Xenopus oocytes of a novel protein, \( p_{Cl}\text{in} \), activated a chloride conductance. We have cloned analogs of \( p_{Cl}\text{in} \) from rat heart and Xenopus ovary. \( p_{Cl}\text{in} \) was identified as an abundant soluble cytosolic protein (~40 kD) that does not immunolocalize with the plasma membrane. \( p_{Cl}\text{in} \) was found in epithelial and cardiac cells, brain, and Xenopus oocytes, forming complexes with soluble actin and other cytosolic proteins. Monoclonal antibodies recognizing \( p_{Cl}\text{in} \) blocked activation of a native hypotonicity-induced chloride conductance \( (I_{Cl,swell}) \) in Xenopus oocytes, suggesting that \( p_{Cl}\text{in} \) may link actin-bound cytoskeletal elements to an unidentified volume-sensitive chloride channel. The high degree of sequence conservation and widespread expression of \( p_{Cl}\text{in} \) suggest that it is an important element in cellular volume regulation.

Introduction

The ability to regulate cell volume precisely is a fundamental property of cells (Chamberlin and Strange, 1989; Hoffmann and Simonsen, 1989; Lewis and Donaldson, 1990; Sarkadi and Parker, 1991). Although the phenomenon of regulatory volume decrease (RVD), whereby a swollen cell loses salt and water to restore its original volume, has been appreciated for decades, the molecular identities of the proteins responsible for the volume control machinery and their regulation are essentially unknown. It appears that the rate-determining step in volume control involves the activation of potassium and chloride conductance pathways (Hoffmann and Simonsen, 1989). At present, however, no potassium channel proteins have been demonstrated to be volume regulated, and only two proteins (P-glycoprotein and CIC-2) have been proposed as swelling-induced chloride channels. Valverde et al. (1992) reported that in NIH3T3 fibroblasts overexpressing P-glycoprotein, cell swelling activated a chloride current. P-glycoprotein behaves as an ion channel independent of its transporter activity (Gill et al., 1992). CIC-2, a ubiquitous channel protein cloned from rat brain, gave rise to a chloride current activated by either strong hyperpolarization when expressed in oocytes (Thiemann et al., 1992) or extracellular hypotonicity (Grunder et al., 1992). However, the CIC-2-expressed chloride current differs in voltage sensitivity, anion selectivity sequence, and pharmacologic sensitivity from swelling-induced chloride conductances characterized in airway epithelia (Kunzelmann et al., 1989; McCann et al., 1989; Chan et al., 1992; Solc and Wine, 1991), oocytes (Ackerman et al., 1994), myocytes (Sorota, 1992; Tseng, 1992), lymphocytes (Lewis et al., 1993), and bovine chromaffin cells (Doroshenko and Neher, 1992).

A third protein that may participate in volume regulation via chloride conductance is \( p_{Cl}\text{in} \). Injection of cRNA encoding Madin–Darby canine kidney (MDCK) \( p_{Cl}\text{in} \) into Xenopus oocytes produced a prominent chloride current (Paulmichl et al., 1992) displaying features consistent with swelling-induced chloride currents seen in colonic epithelia (T84) (Worrell et al., 1989), primary culture epithelial cells (Yan-tomo et al., 1992), human epithelial cells (Kubo and Okada, 1992), airway epithelial cells (Kunzelmann et al., 1989; Solc and Wine, 1991; Chan et al., 1992; McCann et al., 1989), and atrial (Sorota, 1992) and ventricular (Tseng, 1992) myocytes. The \( p_{Cl}\text{in} \)-associated current was elicited in the absence of any osmotic challenge. Although the primary structure deduced from \( p_{Cl}\text{in} \) cDNA did not possess any putative transmembrane domains, \( p_{Cl}\text{in} \) was proposed to make a channel by dimerizing and forming a β barrel pore (Paulmichl et al., 1992). An identical chloride current was not observed in ~5% of water-injected oocytes, suggesting that the \( I_{Cl}\text{in} \)-like channel protein existed endogenously in some oocytes. Subsequently, Ackerman et al. (1994) established that this chloride current could be induced by hypotonicity in 99% of native uninjected oocytes. Figure 1 demonstrates that the native \( I_{Cl}\text{in} \)-like current activated during cell swelling. Previous work has established that the chloride hypoactively-activated current is conducted through chloride channels based on selectivity \( P_{Cl}/P_{Na} > 3 \) and sensitivity to chloride channel blockers (Ackerman et al., 1994).

Further characterization of the protein \( p_{Cl}\text{in} \) was necessary to understand its molecular properties and to identify its relationship to volume-regulated chloride conductances. We report here that an \( I_{Cl}\text{in} \) monocular antibody, which specifically recognized endogenous oocyte \( p_{Cl}\text{in} \), completely prevented the endogenous swelling-induced chloride current when injected into Xenopus oocytes. We have cloned the \( p_{Cl}\text{in} \) homolog from both Xenopus oocytes and cardiomyocytes, using immunoscreening with anti-\( I_{Cl}\text{in} \) antibody and DNA hybridization screening strategies, respectively. Examination of the properties of \( p_{Cl}\text{in} \) suggest that \( p_{Cl}\text{in} \) is more likely a key component of channel activation than an ion channel in itself.

Results

Identification and Localization of Endogenous \( p_{Cl}\text{in} \)

Affinity-purified polyclonal antibodies against β-galactosidase-\( p_{Cl}\text{in} \) (MDCK) fusion protein specifically recognized

*The first two authors contributed equally to this work.
Figure 1. Cell Swelling Activates an Endogenous I_{swell}-Like Current
Representative currents from an oocyte bathed in isotonic (220 mosM) solution followed by perfusion of a hypotonic (110 mosM) solution. Oocytes were voltage clamped at −70 mV and 800 ms potential pulses applied from −90 mV to +70 mV in 20 mV increments. Solid bar indicates zero current. After establishing the basal isotonic current, the oocyte was perfused by hypotonic solution for 5 min before recording. Trace is representative of 185 total experiments in which hypotonicity evoked I_{swell} in 99% of all manually defolliculated oocytes (Ackerman et al., 1994).

Table 1. Quantification of pCch in Cells

<table>
<thead>
<tr>
<th>Cell/Tissue</th>
<th>Molecules per Cell</th>
<th>Concentration (nM)</th>
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<tr>
<td>MDCK</td>
<td>2 × 10^8 to 3 × 10^8</td>
<td>200–300</td>
</tr>
<tr>
<td>Heart (cytosol)</td>
<td>2 × 10^8 to 4 × 10^8</td>
<td>200–300</td>
</tr>
<tr>
<td>Oocyte (cytosol)</td>
<td>~ 10^8</td>
<td>200–300</td>
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Estimates of pCch were determined by quantitative immunoblotting of cell extracts using aFpA antibodies (n = 3). Baculovirus-expressed recombinant pCch was used as a standard.

* Cytosol, 70%–80%; nuclei, 10%–20%; microsomes, <5%.

reveals pCch in a wide variety of cells. In addition to its presence in epithelial kidney cells (MDCK), we have found pCch in heart muscle, brain, and in Xenopus oocytes (Figure 2B). Monoclonal anti-I_{Cch} (FChP12) antibody also identified pCch in each of these cells (data not shown). pCch is easily extracted from cells as a soluble protein, as shown by fractionation of MDCK cells into cytosolic, nuclear, and microsomal fractions, followed by immunoblotting of proteins with affinity-purified polyclonal anti-I_{Cch} (aFpA) antibodies. Quantitation of pCch in MDCK cell fractions (Table 1) showed that pCch is largely cytosolic (70%–80%), while 15%–20% of pCch fractionated with nuclei. Less than 5% of pCch was found in the microsomal fraction that contained plasma membrane. pCch is an abundant protein having roughly the same cytosolic concentration (200–300 nM) in MDCK, heart, and oocytes (Table 1). Although we did not precisely determine the cellular compartmentalization of pCch in heart tissue or in Xenopus oocytes, pCch was also easily extracted from these cells after washing with salt solution. We were unable to identify pCch in either heart sarcolemma or Xenopus oocyte microsomes (data not shown).

The cytosolic and nuclear localization of pCch was confirmed by immunofluorescent staining of fixed MDCK cells. Cytosol and nuclei of formaldehyde-fixed cells were immunostained with affinity-purified polyclonal anti-I_{Cch} (aFpA) antibodies (Figure 3). No staining was observed with control immunoglobulins isolated from immune sera after

Figure 2. Recognition of pCch
(A) Antibodies specifically recognize pCch. Antibodies to recombinant I_{Cch}-β-galactosidase fusion protein recognize a single 37 kd polypeptide in native MDCK cells, Sf9 cells infected with I_{Cch} (MDCK)-pBlueBac III recombinant baculovirus, and X. laevis oocytes injected with I_{Cch} cRNA (MDCK). Cells were lysed in buffer A containing 1% Triton X-100 and protease inhibitor cocktail and spun 10 min at 10,000 x g. Extracted proteins (10 μg for MDCK cells, 20 μg for oocyte, and 0.5 μg for Sf9 cells) were subjected to SDS-PAGE, transferred to PVDF film, immunoblotted with purified monoclonal (FChP12, 1.5 μg/ml; lane 1) or affinity-purified polyclonal (0.5 μg/ml; lanes 2–6) antibodies to recombinant I_{Cch}-β-galactosidase fusion protein, and visualized with secondary [125]labeled antibodies. The film was exposed 12 hr.

(B) Western immunoblot analysis reveals pCch in epithelial cells, cardiac muscle, brain, and Xenopus laevis oocytes. Soluble proteins were extracted as described in Experimental Procedures. Protein (10 μg) was separated by SDS-PAGE, transferred to PVDF film, and immunoblotted with polyclonal anti-I_{Cch} antibodies. Heart soluble proteins were partially enriched for pCch by DEAE-Sepharacel chromatography.
depletion of anti-fusion protein antibodies. There was no specific staining of plasma membrane, but low levels of pICN in the plasma membrane may have been masked by the predominant staining of the cytosol. To examine this possibility, living MDCK cells were immunostained with extracellularly added αFPα without prior fixation as well as by immunostaining of permeabilized cells in which the cytosolic proteins had been washed out. No visible immunofluorescent staining of plasma membrane was observed with either approach (data not shown). Therefore, pICN is predominantly a soluble protein localized to the cytosol rather than an integral membrane protein.

Cloning of ICN in Xenopus Oocytes

Figure 2A (lane 5) and Figure 2B (lane 4) demonstrated that oocytes possess an endogenous ICN-like protein (40 kd) recognized by both polyclonal and monoclonal ICN-specific antibodies. The presence of such an abundant (Table 1) endogenous pICN in oocyte was perplexing, because the MDCK pICN was discovered by expression cloning in Xenopus oocytes (Paulin et al., 1992). The oocyte ICN-like homolog was cloned and sequenced to determine whether differences in pICN in these two tissues (MDCK and oocytes) might account for the apparent inactivity of the native pICN in the Xenopus oocyte.

Figure 4. Comparison of ICN Homologs

(A) Predicted primary structure of ICN clones. Amino acid sequences for Xenopus oocyte, rat atria, and dog kidney (MDCK) ICN are aligned, emphasizing the marked conservation of this protein across different species and tissues. Only differences in amino acid composition in comparison with the original MDCK clone are shown in boldface. The numbers in the lower right indicate the number of amino acids predicted by each clone. Boxed regions indicate the clusters of negatively charged residues. Asparagine mark potential sites for tyrosine phosphorylation.

(B) In vitro translation of ICN clones. Vector constructs containing cDNA encoding for MDCK, atrial, and oocyte ICN were used as templates for in vitro translation. The [35S]methionine-labeled protein products were separated by electrophoresis and autoradiographed. The translation product of each clone (MDCK, 37 kd; atrial, 37 kd; oocyte, 40 kd) in this cell-free system corresponded identically to native pICN (Figure 2B).
We immunoscreened a Xenopus laevis ovarian library (Stratagene) with affinity-purified polyclonal anti-Icn antibody and recovered five independent clones all encoding the same protein. One full-length clone contained 1134 bp with an open reading frame of 723 bp. The 241 amino acid protein predicted from this clone shared 74% amino acid identity with the original MDCK Icn clone (Figure 4A). We also obtained a cardiac Icn clone by screening a rat neonatal atrial library by DNA hybridization. The atrial Icn homolog was 93% identical with MDCK Icn (Figure 4A). These clones displayed no significant homology to other known proteins. The most striking feature seen in these three highly conserved clones is the abundance of negatively charged residues, making them highly acidic (predicted pI = 3.8). Of MDCK and cardiac Icn, 22% consisted of either glutamic or aspartic acid residues, while oocyte Icn contained 24% acidic residues. From amino acids 96 to 110 in oocyte Icn, 13 of 15 residues were either glutamic or aspartic acids. In this same region, acidic amino acids comprised 10 of 15 residues in heart Icn and 9 of 15 residues in MDCK Icn. A second cluster (9 of 13) of acidic residues (amino acids 143–155) contained two putative tyrosine kinase phosphorylation sites (Figure 4A). The significance of these acidic regions is uncertain at present.

In vitro translation of the cDNA clones (Figure 4B) encoding MDCK, heart, and oocyte Icn produced proteins having an electrophoretic mobility identical to the endogenous Icn molecules (see Figure 2B). This suggests that the discrepancy between predicted and apparent molecular weight as well as the difference between the apparent molecular weights of oocyte and other Icn proteins result from peculiarities in the primary structure of Icn rather than posttranslational modifications. Thus, cloning of Icn homologs shows the highly conserved nature of this protein but does not yield clues to the function of native oocyte Icn.

\textbf{Icn Forms Multiple Oligomeric Complexes with Cytosolic Proteins}

Given that Icn is a soluble protein with a predominantly cytosolic localization, the possibility was raised that Icn may be a channel regulator rather than a channel-forming protein, as was initially proposed (Paulovich et al., 1992). To extend our understanding of properties of Icn, we attempted to purify endogenous Icn protein from MDCK cells and heart cytosolic extracts, utilizing ion-exchange chromatography (DEAE), gel filtration, and adsorption (hydroxylapatite and immobilized reactive dye) chromatography. These separation methods revealed several Icn-containing peaks in their elution profiles. After DEAE–Sepharose chromatography, for example, Icn eluted as at least three discrete molecular species during size-exclusion chromatography (Figure 5A). Nondenaturing electrophoresis of Icn partially purified from dog heart cytosolic protein extracts revealed multiple (at least four) distinct complexes of Icn (Figure 5B). Lane 1 of Figure 5B shows that overexpressed recombinant S9 Icn is a monomer under nondenaturing conditions, while native heart cytosolic Icn forms several molecular complexes (apparent molecular mass 300 kd, 90 kd, 73 kd, and 48 kd; Figure 5B, lane 2). In contrast, in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), endogenous Icn migrated as a single polypeptide (see Figures 2A and 2B). Taken together, these data suggest that Icn may form homomultimers or interact with other cytosolic proteins. However, since the molecular weights of Icn oligomers under nondenaturing conditions are inconsistent with homomultimeric forms of this molecule, we tested whether Icn forms multiple complexes with other cytosolic proteins.

Icn was immunoprecipitated with affinity-purified polyclonal anti-Icn antibodies from MDCK cells metabolically labeled with $^{35}$S methionine to reveal the components of the Icn oligomeric complexes. Several polypeptides (molecular masses, 90 kd, 80 kd, 72 kd, 43 kd, and 29 kd)
Figure 6. Cytosolic Proteins Interact with pLcm
(A) Coinmunoprecipitation of MDCK cell cytosome proteins in complexes with pLcm. Cytosome proteins from ^[35]S)methionine-labeled MDCK cells were immunoprecipitated with anti-pLcm antibodies (aFPA). Immunoprecipitated proteins were separated on an SDS-PAGE and visualized by fluorography of the dried gel.

Lanes 2–4 display the pattern of cytosome proteins isolated from MDCK, heart, and oocyte interacting with pLcm by affinity chromatography on immobilized recombinant pLcm revealed by silver staining of proteins after SDS–PAGE.

(B) Specificity of binding of heart cytosome proteins to immobilized recombinant pLcm. Dog heart cytosome proteins (lane 1) were affinity isolated on either immobilized recombinant pLcm (lane 2), immobilized and boiled recombinant pLcm (lane 3), or immobilized BSA (lane 4). Two abundant proteins that interacted with pLcm (p43 and p72) could be competed off the pLcm affinity column with 0.15 mg/ml free recombinant pLcm (lane 5) but not with 0.15 mg/ml free BSA (lane 7). Lane 6 shows that the purified recombinant pLcm used for the competition was not contaminated with either p43 or p72.

were always observed to coinmunoprecipitate with pLcm (Figure 6A, lane 1) but never immunoprecipitated with control antibodies (data not shown). These polypeptides are candidates for plcm–associating proteins that comprise the tight complexes shown in Figure 5. The most prominent cytosome polypeptides interacting with pLcm were p43 and p72.

To confirm the specificity of protein–pLcm interactions and to purify and identify these proteins, we designed a pLcm-affinity column. For this purpose, MDCK pLcm was overexpressed in S9 cells by a recombinant pLcm–baculovirus construct, purified using two step chromatography (see Experimental Procedures), and covalently bound to w-aminobutyyl agarose. We incubated cytosome extracts with affinity resin overnight to allow immobilized pLcm to replace endogenous pLcm in oligomeric complexes. The polypeptides (p90, p80, p72, and p43) from MDCK cytosome that were tightly bound to immobilized pLcm (Figure 6A, lane 2) coincided with the coinmunoprecipitated polypeptides (lane 1). The coincidence of affinity-isolated and coinmunoprecipitated polypeptides from MDCK cytosome suggests that these proteins specifically interact with pLcm.

We then affinity-isolated cytosome proteins from heart and oocyte that specifically interacted with immobilized pLcm. Two proteins, p43 and p72, were consistently isolated regardless of the source of protein (kidney, heart, or oocyte). Other source-specific minor proteins were observed after affinity isolation on immobilized pLcm (Figure 6A). As a control, no protein binding was observed after overnight incubation of cytosome extracts with bovine serum albumin (BSA)-immobilized resin (Figure 6B, lane 4). Additional evidence pertaining to the specificity of pLcm–cytosolic protein interaction was provided by competition of free and bound pLcm (Figure 6B, lane 5). Free BSA was unable to compete p43 or p72 from the immobilized pLcm column (Figure 6B, lane 7). Figure 6B (lane 6) verifies that the purified pLcm used for immobilization as well as competition was not contaminated with either p43 or p72. Interestingly, these same proteins interacted not only with native immobilized pLcm (Figure 6B, lane 2) but also with denatured immobilized pLcm that was either boiled (lane 3) or treated with SDS (data not shown).

43 kd Protein Interacting with pLcm Is Actin
The size of the 43 kd cytosome protein interacting with pLcm and the suggestion that actin filaments (43 kd monomer
Figure 8. Injection of Anti-pLcn Antibody into Xenopus Oocytes Blocks the Swelling-Induced Chloride Current, ICswell
(A) Anti-pLcn antibody blocks ICswell in Xenopus oocytes. Representative current traces from three separate oocytes injected with either 100 mM KCl, 2.5 mg/ml unrelated IgG, monoclonal antibody, or 0.1 mg/ml anti-Lcn. FCHP12 monoclonal antibody (50 nl injection volume). Traces from representative recordings are superimposed for comparison. The swelling-induced chloride current was measured using two electrode voltage clamp. At 22–24 hr after injection, ICswell was elicited after 5 min exposure to the hypotonic solution by putting to +70 mV for 800 ms from a holding potential of −70 mV. Dashed line indicates zero current. Note that both the outward chloride current at +70 mV and the inward chloride current at the holding potential were markedly suppressed in the anti-pLcn antibody–injected oocyte.

(B) Summary of anti-Lcn antibody block of ICswell in Xenopus oocytes. Oocytes were injected with 50 nl of purified monoclonal antibody FCHP12 (0.1 mg/ml, n = 6; 0.5 mg/ml, n = 4) that specifically recognized endogenous oocyte Lcn. KCl (100 mM; n = 9) and an unrelated IgG, monoclonal antibody (2.5 mg/ml, n = 8) were injected as separate controls. The magnitude of ICswell was obtained by measuring the outward current elicited at 30 ms during a pulse to +70 mV as in (A) and subtracting 200 nA to remove the average lanthaun-insensitive current that reflects a basal conductance other than ICswell (Ackerman et al., 1994). Data are represented as mean ± SEM.

(C) Time course of inhibition of ICswell by anti-pLcn antibody. Oocytes were injected with either 5 ng (0.1 mg/ml) anti-Lcn antibody or unrelated IgG, monoclonal antibody. Peak ICswell current was measured every 4 hr (n > 4 each set). The magnitude of ICswell in antibody-injected oocytes was normalized with the current elicited in KC1-injected (n > 4) oocytes at each time period. Data are presented as mean ± SEM.

pLcn Is Critical for the Activation of Swelling-Induced Chloride Currents in Xenopus Oocytes

Xenopus oocytes possess both an Lcn protein highly homologous with MDCK pLcn and a native swelling-induced chloride current (ICswell) (Ackerman et al., 1994) indistinguishable from the heterologously (MDCK Lcn) expressed chloride current in oocytes (Paulmichl et al., 1992) (see Figure 1). Two experimental approaches were tested to determine whether endogenous pLcn and the endogenous hypotonicity-activated chloride current are related. First, the Xenopus oocyte Lcn DNA sequence was used to generate Lcn antisense oligonucleotides that were injected into oocytes. Unfortunately, the time course of antisense-induced pLcn depletion overlapped with the slow decline of the swelling-induced chloride current in control oocytes maintained in artificial media for several days (discussed in Ackerman et al., 1994). Therefore, it was impossible to ascertain the effect of pLcn depletion on the swelling-induced chloride conductance in oocytes by this method. In the second approach, we injected oocytes with monoclonal anti-Lcn antibody (FCHP12), which specifically recognized only the pLcn polypeptide. Figure 8 summarizes the results of these experiments.

The injection of monoclonal anti-Lcn antibody completely suppressed the swelling-induced chloride current (ICswell) at 22–24 hr, compared with matched controls (Figures 8A and 8B); 5 ng of monoclonal antibody attenuated ICswell by 90% (n = 8), and 25 ng of anti-Lcn antibody (n = 4) almost completely eliminated ICswell, as compared with ICswell elicited in oocytes injected with vehicle alone (100 mM KCl, n = 9). The action of anti-Lcn antibody (FCHP12) appears to be specific for FCHP12-pLcn interaction for two reasons.
First, 5 ng of injected antibody (33 nM final oocyte concentration) approximates the \( K_d \) for this antibody–antigen complex (20 nM according to enzyme-linked immunosorbent assay; data not shown). Second, an unrelated IgG at a 25-fold higher concentration did not significantly inhibit \( I_{C_{swat}} \) (\( n = 8 \)). Figure 8C shows the time course of \( I_{C_{swat}} \) suppression after injection of monoclonal antibody. The half-maximal suppression by 5 ng of FChP12 occurred approximately 12 hr following antibody injection. Increasing the concentration of injected antibody by 25-fold resulted in only a modest increase (approximately 2-fold) in the rate of \( I_{C_{swat}} \) inhibition (data not shown). This suggests that the interaction between FChP12 and \( p_{cln} \) is not the rate-limiting step for the attenuation of \( I_{C_{swat}} \). The slow kinetics of \( I_{C_{swat}} \) inhibition parallels the slow time course of \( p_{cln} \) immunoprecipitation by FChP12 observed under non-denaturing conditions in preliminary experiments and contrasts with the rapid immunoprecipitation of \( p_{cln} \) noted under denaturing conditions. One explanation of this result is shielding of the epitope by associated cytosolic proteins in vivo. Given the specificity of monoclonal antibody (FChP12) interaction with oocyte \( p_{cln} \) and the complete inhibition of \( I_{C_{swat}} \) by this antibody, we conclude that endogenous oocyte \( p_{cln} \) is critical for the activation of the endogenous swelling-induced chloride current.

**Discussion**

\( p_{cln} \) was originally cloned using expression of a novel chloride current in Xenopus oocytes. The simplest interpretation of the data was that \( p_{cln} \) was in itself a channel. The lack of homology to known channels necessitated that a novel dimeric \( \beta \) barrel structure be proposed (Paulinichl et al., 1992). The investigation of molecular properties of native \( p_{cln} \) in mammalian cells and oocytes demonstrated that this protein is common and abundant. Cell fractionation and immunolocalization revealed that this protein is cytosolic, with <5% in the microsomal fraction tightly associated with cytoskeleton elements. \( p_{cln} \) was not detected in the plasma membrane, although a small percentage of membrane-bound \( p_{cln} \) cannot be ruled out. None of the described properties of \( p_{cln} \) appear to be consistent with fundamental features of channel-forming proteins. Furthermore, we did not detect chloride channel activity in patch-clamped S9 cells overexpressing \( p_{cln} \) (\( n = 86 \)). In addition, no channel activity was detected in lipid bilayers (\( n = 50 \)) or in giant liposomes (\( n = 65 \)) reconstituted with highly purified \( p_{cln} \) (data not shown).

It is possible that soluble \( p_{cln} \) molecules are in dynamic equilibrium with channel-forming membrane-bound dimeric \( p_{cln} \) or that the mechanism of activation of swelling-induced ion channels involves the insertion of new channels contained within cytoplasmic vesicles in response to a volume perturbation (Foskett and Spring, 1985). In this case, the dynamic equilibrium between the soluble and the channel-forming state of \( p_{cln} \), may shift toward the membrane-bound channel form of \( p_{cln} \) under hyponotic conditions. However, this model seems unlikely, because \( p_{cln} \) could not be detected in the plasma membrane by immunocytochemistry after incubating the cells in a hyponotic media.

\( p_{cln} \) exists primarily in the cytosol in tight specific complex formations with other cytosolic proteins. Interestingly, these protein–protein interactions occur in vitro even after \( p_{cln} \) is denatured by boiling or SDS treatment. These features are reminiscent of at least two well-known regulatory systems: multiple targeting complexes of calmodulin (Binglinsley et al., 1990) and growth factor–dependent phosphorylation cascades where regulatory molecules like ras (Moodie et al., 1993), GRB2 (Chard et al., 1993; Egan et al., 1993), and IRS-1 (Myers and White, 1993) physically associate with other molecules through a primary sequence determined by SH2 and SH3 domains. The most prominent characteristic of the \( p_{cln} \) primary structure is the presence of two extremely acidic and highly conserved domains. Conceivably, these regions may be important in mediating regulatory protein–protein interactions similar to the acidic activation domains found in eukaryotic transcription factors (Ptashne, 1988; Swanson et al., 1991; Li and Botchan, 1993). Based upon the properties of native \( p_{cln} \) protein, the proposal that \( p_{cln} \) is a channel regulator rather than an ion channel is the simplest interpretation of the data.

The original expression cloning of MDCK \( I_{C_{swat}} \) in oocytes that possess \( p_{cln} \) endogenously may have been accomplished via an alteration of \( p_{cln} \) in molecular complexes. If \( p_{cln} \) is an activator of \( I_{C_{swat}} \), its intrinsic activity may be suppressed by associated proteins. In this case, overexpression of exogenous MDCK \( p_{cln} \) in Xenopus oocytes may lead to the activation of endogenous chloride channels. Alternatively, one of the proteins that interact with \( p_{cln} \) may be a negative modulator of \( I_{C_{swat}} \). In this case, overexpression of \( p_{cln} \) in oocytes might disinhibit \( I_{C_{swat}} \) channels by binding to the inhibitory protein.

We have established that endogenous \( p_{cln} \) is critical for the activation of an endogenous swelling-induced chloride current \( (I_{C_{swat}}) \) in oocytes because perturbations of the intracellular activities of \( p_{cln} \) by monoclonal anti-pcln antibodies abolished \( I_{C_{swat}} \). The mechanisms underlying the regulation of volume-sensitive chloride channels are essentially unknown. It has been proposed that changes in the concentration of cytosolic regulatory proteins (Jennings and Schulz, 1990) as well as changes in cytoskeleton elements (Mills, 1987) may couple disturbances in cell volume with modulation of ion channels (reviewed in Sarkadi and Parker, 1991). The identified protein–protein interaction between \( p_{cln} \) and soluble actin and the recognition of cytoskeletal-associated \( p_{cln} \) makes \( p_{cln} \) a candidate protein of such regulatory schemes.

The finding that native \( p_{cln} \) in oocytes is critical for the activation of a native swelling-induced chloride conductance pathway establishes an unambiguous link between a cloned protein and a specific volume control mechanism. Considering that epithelial and cardiomyocyte \( I_{C_{swat}} \) are virtually the same as oocyte \( I_{C_{swat}} \), we speculate that \( p_{cln} \) behaves in an analogous fashion in epithelial cells and cardiomyocytes. Future studies revealing the identities of the cytosolic proteins that interact with \( p_{cln} \) are required to understand the mechanism behind the regulation by
of these volume-sensitive chloride currents. Moreover, if pIC50 is in fact a swelling-induced chloride conductance regulator rather than a channel protein, then the molecular identity of the hypotonicity-activated chloride channel in oocytes, myocytes, and epithelia remains unknown. Although two cloned proteins, P-glycoprotein (Valverde et al., 1992) and CIC-2 (Grunder et al., 1992), have been identified as candidates for volume-sensitive chloride channels, neither protein appears to be responsible for lumen in these cells (Ackerman et al., 1994). It has often been proposed that alternate conductance pathways in airway epithelia could be used to bypass the defective chloride conductance in cystic fibrosis patients. If so, a regulatory mechanism for the swelling-induced chloride channel is a potential target for new therapeutic agents.

Experimental Procedures

Isolation and Characterization of Anti-lumen Antibodies

β-Galactosidase-lumen (MDCK) fusion protein was used as antigen for obtaining polyclonal (rabbit) and monoclonal antibodies to pIC50. lumen (MDCK) cDNA was cloned into the pAX5+ vector (USB), and β-galactosidase-lumen (MDCK) fusion protein was generated in the bacterial host XL1-Blue according to the protocol of the manufacturer. The fusion protein was purified by ammonium sulfate precipitation of bacterial lysate, followed by affinity purification of fusion protein on an APTG affinity column (Ullmann, 1984). Recombinant pIC50 was cleaved from the β-galactosidase-lumen (MDCK) fusion protein by factor Xa (New England Biolabs) and purified by chromatography on an APTG affinity column and Mono Q (Pharmacia) as a protein with electrophoretic mobility corresponding to 37 kDa. From the N-terminus of this protein, 15 residues were microsequenced and matched precisely with the predicted amino acid sequence of MDCK lumen cDNA. This recombinant lumen protein was used for initial characterization of anti-lumen sera and selection of hybridomas producing anti-lumen antibodies.

Anti-pIC50 polyclonal antibodies were purified in two steps. First, sera were depleted of anti-β-galactosidase antibodies by passing over immobilized bacterial β-galactosidase. Bacterial β-galactosidase was produced and purified in the same manner as the fusion protein. Immobilization was achieved by covalently binding protein to CNBr-activated Sepharose (Sigma). Second, anti-lumen antibodies were affinity purified from β-galactosidase-depleted serum on a column with immobilized β-galactosidase-lumen (MDCK) fusion protein in the same manner. These affinity-purified polyclonal immunoglobulins (aFPA) recognized 0.5 ng of purified recombinant pIC50 (baculovirus) on Western blot at a concentration of 0.5 μg/mL. Three hybridomas producing monoclonal anti-lumen antibodies were obtained: IgG1 (FChP12), IgG (FChP 6 and 8), and FChP12, which was purified from ascitic fluid by protein G affinity chromatography. FChP12 recognized 10 ng of purified recombinant pIC50 on Western blot at a concentration of 1.5 μg/mL. Only aFPA and the monoclonal antibody FChP12 were used in this investigation.

Cells, Tissues, and Protein Extraction

MDCK cells were grown in MEM (Gibco) supplemented with 10% FCS in 5% CO2. For soluble protein extraction, confluent cells were washed with PBS, scraped, and desnuced homogenized (3 strokes, pestle A) in MB (10 mM K/Hepes, 20 mM KCl, 1 mM EGTA, 3 mM MgCl2, 1 mM DTT [pH 7.4]) containing protease inhibitor cocktail (PIC, Sigma): 2 μg/mL aprotime, leupeptin, and pepstatin A, and 0.5 mM PMSF. After centrifugation (40,000 × g for 30 min), the supernatant containing soluble proteins was isolated. MDCK cells were lysed in MB supplemented with 250 mM sucrose and PIC and fractionated by step centrifugation for 10 min at 13000 × g. The pellet was rehomogenized and sedimented again under the same conditions to yield cellular nuclei. Pooled 13000 × g supernatants were centrifuged for 2 hr at 100,000 × g. The resulting pellet and supernatant contained microsomal and cytosolic proteins, respectively.

Xenopus oocyte soluble proteins were recovered from supernatant after homogenization of oocytes in MB (supplemented with the protease inhibitor cocktail) and centrifugation for 30 min at 10,000 × g. Oocyte microsomal proteins were obtained according to the procedure of Colman (1984).

To obtain soluble proteins from dog heart, ventricular muscle was homogenized in MB–PIC using a Polytron (3 × 30 s) followed by centrifugation for 20 min at 14,000 × g. The supernatant was centrifuged for 2 hr at 150,000 × g. pIC50 was partially purified from this soluble protein-containing extract by DEAE–Sephacel (Pharmacia) chromatography. The proteins eluting between 0.1 and 0.45 M NaCl were collected, yielding 100% recovery and a 6-fold purification of pIC50. Further fractionation was done by Sephacryl S200 (2.6 × 90 cm) size-exclusion chromatography in MB–100 mM NaCl with a flow rate of 0.5 ml/min. Sarcoclemma from dog heart was isolated according to Slaughter et al. (1983).

Immunocytochemistry

Following SDS–PAGE (12% gel; Laemmli, 1970) and electrophoretic transfer to PVDF membrane (Millipore), membranes were blocked with 5% powdered milk in TBS, 0.1% Tween 20 overnight. Membranes were then incubated with primary antibodies (polyclonal aFPA, 0.5 μg/mL or monoclonal FChP12, 1.5 μg/mL) diluted in the blocking solution with 3% powdered milk for 1 hr. After washing with TBS, 0.1% Tween 20, membranes were incubated in the blocking solution with 3% powdered milk for 1 hr with proper secondary antibodies (145)-labeled anti-immunoglobulin G Fab (New England Nuclear). Washed and dried membranes were exposed overnight on X-ray film.

For quantitative immunoblotting of pIC50 in cell/tissue extracts, aFPA was used as the primary antibody, and purified baculovirus-expressed recombinant pIC50 was used as the standard.

Monoclonal anti-actin antibodies (Amersham) were used for identification of actin.

Nondenaturing electrophoresis was done in a 10%–30% gradient gel in a Tria–acetate (pH 6.4) system using a Tria–alanine (pH 6.8) electrode buffer (adapted from a protocol provided by Pharmacia). After protein separation, gels were soaked in 1% SDS solution for 30 min, and proteins were transferred to the PVDF membrane using a semi-dry transfer apparatus (Hofer Scientific Instruments).

Immunoprecipitation

Confluent cultures of MDCK cells were incubated in methionine-depleted MEM (Gibco) supplemented with 5% FCS for 30 min. Cells were metabolically labeled for 12 hr with [35S]methionine (Amersham) at 50 μCi/ml in a methionine-free medium. After washing with cold PBS, cells were denatured homogenized in MB containing PIC followed by centrifugation for 30 min at 40,000 × g. Extracted cytosolic proteins were concentrated by Centricon-10 (Amicon), and 200 μL (0.5 mg) was used for immunoprecipitation. After preincubating with 70 μL of protein A-Sepharose Fast Flow (Pharmacia), the concentrate was incubated with 30 μg of aFPA for 2 hr at 4°C. Immune complexes were recovered by mixing with 20 μL of protein A-Sepharose Fast Flow for 2 hr at 4°C. The immunoprecipitate was washed five times with 1 ml of solution containing 10 mM Tris (pH 7.4), 0.4 M NaCl, and 1% Triton X-100. Immunoprecipitated proteins were resolved by SDS–PAGE and visualized by fluorography of the dried gel.

Immunocytochemistry

MDCK cells were grown on coverslips to approximately 30% confluency, fixed in solution containing 3% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Cells were blocked with 3% BSA for 1 hr and then incubated with affinity-purified polyclonal (1.5 μg/mL) anti-lumen antibody at room temperature for 1 hr. Cells were then incubated for 1 hr with FITC-conjugated anti-rabbit antibody (Cappell). Immunoglobulins isolated from anti-lumen immune serum, which had been depleted of anti-β-galactosidase and anti-lumen antibodies, were used as a control. Cells were imaged using a Nikon Microphot-FXA fluorescence microscope (Nikon Plan-apo neofluor objective, 60 x). Images were recorded on Hypertech film (Microfluor, Limited).

Purification of Baculovirus- or Sf9-Expressed Recombinant pIC50

The MaxBac baculovirus expression system (Invitrogen) was utilized for the production of a recombinant nonfusion lumen protein from the MDCK cDNA clone. The coding region of lumen (MDCK) was unidirectional-
ally ligated into the baculovirus expression vector, pBlueBac Iii. This construct was cotransfected with linear wild-type baculovirus DNA into Sf9 cells. Recombinant baculovirus constructs were detected by the coexpression of β-galactosidase activity. Recombinants containing LCl sequence were further characterized by PCR analysis.

Cultures of Sf9 cells in spinner flasks were infected with the recombinant pLCl baculovirus at a multiplicity of 5:1 (virus:Sf9 cells) and incubated for 72 hr. Soluble proteins from Sf9 cells were extracted by homogenization cells in buffer A (10 mM HEPES [pH 7.3], 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 0.8 mM Benzamidene). Supernatant extract (100,000 × g) was combined with the cell medium. Soluble protein (120 mg) was applied on a DEAESephacel column (100 ml) equilibrated with 200 mM NaCl in Buffer A. The recombinant LCl protein was eluted with an increasing salt gradient from 200 mM to 450 mM NaCl. pLCl-containing fractions were determined by Western blot and pooled. This sample was concentrated by ultrafiltration, applied to a Sephacryl S200 HR sizing column (2.6 × 90 cm), and eluted with Buffer A (excluding DTT), containing 100 mM NaCl. A 500 ml culture yielded approximately 2 mg of purified LCl protein.

Purified recombinant pLCl was covalently bound to α-aminoxybutyl agarose (Sigma) with sulfos-SMBP (Pierce) according to the protocol of the manufacturer. The affinity matrix contained 0.3–0.5 mg of bound pLCl per ml resin. For affinity isolation of proteins interacting with pLCl, soluble proteins (3–4 mg in 0.5 ml of MB containing 400 mM NaCl and 0.1% Lubrol PX) were rotated overnight at 4°C with 100 μl affinity resin. After removal of unbound proteins by washing five times with 1 ml of each buffer, bound proteins were eluted by boiling in SDS sample buffer.

Cloning of LCl. Homologs from Xenopus Oocytes and Neonatal Rat Atria

An ovarian cDNA library from Xenopus laevis (Unizap, Stratagene) was probed for an LCl analog by immunoscreening. The phage cDNA library (5 × 10^6 plaque-forming units) was screened, and expression of the library-encoded β-galactosidase fusion proteins was induced with IPTG. Expression of the cDNA was then transferred to nitrocellulose and probed with a polyclonal anti-LCl antibody (aFPA). A secondary antibody, HRP-conjugated goat anti-rabbit antibody, was used in conjunction with an enhanced chemiluminescence (ECL) detection system (Amersham) for identification and localization of positive plaques. Plasmids containing the positive clones were rescued from the phage, and their DNA sequences were determined. Five independent clones were isolated that all encoded the same protein.

For the cloning of a cardiac version of LCl, a neonatal rat atria cDNA library was constructed from 5 μg of polyadenylated RNA obtained from 65 neonatal rat atria, using the Unizap kit from Stratagene. Plaques (5 × 10^5) were screened by nucleic acid hybridization (Quik-Hyb; Stratagene) using a randomly primed 700 bp PCR product. This PCR product was amplified from the atrial tissue using primers specific to the N- and C-termini from the coding region of MDCX LCl. A unique full-length clone was isolated and sequenced in both directions with T7 DNA polymerase (US Biochemical).

In Vitro Translation

DNA (1 μg) from each of the vector constructs containing MDCX, rat atria, and Xenopus oocyte LCl was used for in vitro translation with the TNT Coupled Reticulocyte Lysate System (Promega). The [35S] methionine-labeled protein products were run on a 10% discontinuous polyacrylamide electrophoresis gel and autoradiographed.

Electrophysiology

The swelling-induced chloride current (I_{swell}) was elicited from Xenopus oocytes as shown by Ackerman et al. (1994). Briefly, stage V–VI oocytes were manually defolliculated within 2 hr after their surgical removal from anesthetized Xenopus laevis tadpoles and immediately (≤ 2 hr) injected with either anti-LCl monoclonal antibody (FCHP12), anti-LCl polyclonal antibody (aFPA), unrelated monoclonal IgG antibody, exhausted polyclonal antibodies, or KCl. All antibodies were dialyzed against 100 mM KCl. The injected oocytes were allowed to incubate for 4–24 hr at 19°C in ND96 (220 mosM solution containing the following: 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES [pH 7.5]). The hypotonicity-activated chloride current was then measured using the two-electrode voltage clamp technique with a Turbo TEC O1C from NPI Instruments, Tamm, Federal Republic of Germany. Oocytes were voltage clamped at a holding potential of −70 mV 5 min following perfusion of a hypotonic (110 mosM) solution (ND48) containing the following: 48 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES (pH 7.5). An 800 ms depolarizing pulse to −70 mV was applied, and the current's amplitude at 30 ms was measured. The lanthanum-insensitive component (approximately 200 nA) was subtracted to obtain I_{swell}. (Ackerman et al., 1994).

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GenBank Accession Numbers

The accession number for the toad sequence reported in this paper
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Note Added in Proof

Using antisense oligodeoxynucleotides, a direct link of ICa, and the
swelling-induced chloride current in NIH3T3 fibroblasts has been
shown (Gschwendner, M., Nagl, O. U., Woll, E., Schmarda, A., Ritter,
cell volume–induced activation of chloride channels [submitted].)