Recombinant pI<sub>Cln</sub> Forms Highly Cation-selective Channels when Reconstituted into Artificial and Biological Membranes

**Canhui Li, Sylvie Breton, Rebecca Morrison, Carolyn L. Cannon, Francesco Emma, Roberto Sanchez-Olea, Christine Bear, and Kevin Strange**

From the *Division of Cell Biology, Research Institute, Hospital for Sick Children and Physiology Department, University of Toronto, Toronto, Ontario, Canada M5G 1X8; 1Renal Unit, Massachusetts General Hospital, Boston, Massachusetts 02129; 2Anesthesiology Research Division, Department of Anesthesiology and Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and Children’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

**ABSTRACT** pI<sub>Cln</sub> has been proposed to be the swelling-activated anion channel responsible for I<sub>Cl,swell</sub> or a channel regulator. We tested the anion channel hypothesis by reconstituting recombinant pI<sub>Cln</sub> into artificial and biological membranes. Single channels were observed when pI<sub>Cln</sub> was reconstituted into planar lipid bilayers. In the presence of symmetrical 300 mM KCl, the channels had a high open probability and a slope conductance of 48 pS, and were outwardly rectifying. Reduction of trans KCl to 50 mM shifted the reversal potential by -31.2 ± 0.06 mV, demonstrating that the channel is at least seven times more selective for cations than for anions. Consistent with this finding, channel conductance was unaffected by substitution of Cl<sup>-</sup> with glutamate, but was undetectable when K<sup>+</sup> was replaced by N-methyl-D-glucamine. Reconstitution of pI<sub>Cln</sub> into liposomes increased 86Rb<sup>+</sup> uptake by three- to fourfold, but had no effect on 36Cl<sup>-</sup> uptake. Phosphorylation of pI<sub>Cln</sub> with casein kinase II or mutation of G54, G56, and G58 to alanine decreased channel open probability and 86Rb<sup>+</sup> uptake. When added to the external medium bathing Sf9 cells, pI<sub>Cln</sub> inserted into the plasma membrane and increased cell cation permeability. Taken together, these observations demonstrate that channel activity is due to pI<sub>Cln</sub>, and not minor contaminant proteins. However, these findings do not support the hypothesis that pI<sub>Cln</sub> is the anion-selective I<sub>Cl,swell</sub> channel. The observed cation channel activity may reflect an as yet to be defined physiological function of pI<sub>Cln</sub>, or may be a consequence of in vitro reconstitution of purified, recombinant protein.

**KEY WORDS:** cell volume • swelling-activated anion channels • planar lipid bilayer • liposomes • recombinant protein

**INTRODUCTION**

An apparently ubiquitous response to swelling in vertebrate cells is activation of an outwardly rectifying anion current termed I<sub>Cl,swell</sub>. The general characteristics of this current include an Eisenman type I anion permeability sequence (I<sup>-</sup> > Br<sup>-</sup> > Cl<sup>-</sup> > F<sup>-</sup>), modest outward rectification, voltage-dependent inactivation at potentials above E<sub>Cl</sub>, inhibition by a wide variety of compounds including conventional anion transport inhibitors, and block by extracellular nucleotides (Strange et al., 1996; Okada, 1997).

Molecular identification of the protein(s) giving rise to I<sub>Cl,swell</sub> has been controversial and confusing (Strange et al., 1996; Nilius et al., 1997; Okada, 1997; Strange, 1998). Gill et al. (1992) and Valverde et al. (1992) proposed that P-glycoprotein, the product of the multidrug resistance 1 gene, functions as both a drug transporter and the I<sub>Cl,swell</sub> channel. However, numerous laboratories have been unable to reproduce the findings of these investigators, and additional experimental observations have failed to support the original hypothesis (reviewed by Wine and Luckie, 1996; Okada, 1997). Subsequently, it was suggested that P-glycoprotein functions to modulate or regulate I<sub>Cl,swell</sub> (reviewed by Wine and Luckie, 1996; Okada, 1997). It is not clear whether the apparent modulation of I<sub>Cl,swell</sub> by P-glycoprotein reflects a physiologically relevant function, or whether it simply a consequence of overexpression of the protein induced by transfection or drug selection.

pI<sub>Cln</sub> is a ubiquitous and abundant 27-kD soluble protein that is localized primarily to the cytoplasm (Krapivinsky et al., 1994; reviewed by Strange, 1998). Because of its ability to induce in Xenopus oocytes an outwardly rectifying anion current that superficially resembles I<sub>Cl,swell</sub>, pI<sub>Cln</sub> has been proposed to be either the I<sub>Cl,swell</sub> channel (Paulmichl et al., 1992; Gschwentner et al., 1995) or a channel regulator (Krapivinsky et al., 1994). However, as with P-glycoprotein, key observations supporting these hypotheses have not been reproduced or supported by additional experimental evidence, and
the role of pICln in I\textsubscript{Cl, swell} channel function, if any, remains uncertain (reviewed by Strange, 1998).

To test the hypothesis that pICln is an anion channel-forming protein, we reconstituted purified, recombinant protein into artificial and biological membranes. Our results demonstrate conclusively that pICln is capable of generating channel activity in vitro, but the channels formed by the protein are highly cation selective. These findings do not support the hypothesis that pICln is the I\textsubscript{Cl, swell} channel. The observed cation channel activity may reflect an as yet to be defined physiological function of pICln, or it may be a consequence of in vitro reconstitution of recombinant protein. Our findings provide the basis for further physiological investigations of pICln and may provide novel insights into the structure of channel-forming proteins and protein–membrane interactions.

**MATERIALS AND METHODS**

**Production and Purification of Recombinant pICln**

A fusion protein consisting of glutathione S-transferase (GST)\textsuperscript{1} and full length pICln cloned from rat C6 glioma cells was ligated into the pGEX–FK–T vector and expressed in BL21 *Escherichia coli* using a commercially available kit (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The GST–pICln fusion protein was purified from bacterial lysates using glutathione Sepharose 4B. pICln was cleaved from the GST moiety with thrombin, and the thrombin was removed by treatment with benzamidine Sepharose 6B (Pharmacia LKB Biotechnology Inc.). As a control, lysates from bacteria expressing GST alone were subjected to the same purification procedure as the GST–pICln fusion protein.

**Phosphorylation and Mutagenesis of pICln**

Casein kinase II (CKII) was used to phosphorylate pICln (Sanchez-Olea et al., 1998). Approximately 200 μg of GST–pICln was incubated at 30°C with 500 U CKII in 100 μl of reaction buffer (New England Biolabs Inc., Beverly, MA) containing 500 μM ATP. After 30 min, GST–pICln was washed with phosphate-buffered saline and subjected to the cleavage procedure described above.

Glycine residues at positions 54, 56, and 58 of C6 glioma cell pICln (Sanchez-Olea et al., 1998) correspond to G49, G51, and G53 of Madin-Darby Canine Kidney (MDCK) cell pICln (Paulmichl et al., 1992). These residues were mutated to alanine using standard PCR approaches.

**Planar Bilayer Studies of Single Channel Activity**

Planar lipid bilayers were formed by painting a 10 mg/ml solution of phospholipid (phosphatidylethanolamine [PE]:phosphatidylserine [PS] at a ratio of 1:1) in 6-decane over a 200-μm aperture in a bilayer chamber. Lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL), and were >99% pure. The cis compartment of the bilayer chamber is defined as that compartment to which proteoliposomes were added, and the trans compartment is defined as the compartment connected to ground. Bilayer solutions contained 50 or 300 mM KCl and 10 mM 4-nitrophenylphosphonate sulfonyl acid (MOPS), pH 7.0. For ion substitution experiments, KCl was substituted with either N-methyl-d-glucamine (NMDG)-Cl or K-glutamate.

pICln was incorporated by sonication into liposomes comprised of PE:PS:phosphatidylcholine:ergosterol (4:1:5:1 ratio by wt) in 1:10 protein:lipid (by wt) ratio. Lipids were purchased from Avanti Polar Lipids, Inc. and were >99% pure. Proteoliposomes were purified by passing through a Sephadex G-50 gel filtration column (Fisher Scientific Co., Pittsburgh, PA) to remove pICln remaining in solution. To promote and monitor bilayer fusion, proteoliposomes were doped with nystatin (120 μg/ml liposomes) as described originally by Woodbury and Miller (1990). Channel activity was monitored after addition of proteoliposomes to the cis compartment of the bilayer chamber (1 μg protein/ml bilayer solution). Fusion events were detected as transient, nystatin-induced conductance spikes. Single channel currents were measured with a bilayer amplifier (custom made by M. Shen, Physics Lab, University of Alabama, University, AL). Electrical connections between the bath chambers and the amplifier were made using 3 M KCl agar bridges. Data were recorded and analyzed using pCLAMP 6.0.2 software (Axon Instruments Inc., Foster City, CA). Before analysis of dwell times, single channel data were digitally filtered at 100 Hz.

**Histogram Analysis**

Open and closed time histograms were created with a logarithmic x axis with 10 bins/decade and a lower limit of 10 ms. The maximum likelihood method was used to fit the data with one or two exponentials (pCLAMP 6.0.2 software; Axon Instruments Inc.). The “goodness” of fit was assessed using the log likelihood ratio test.

**Concentrative Tracer Uptake Assay for Study of Channel Activity**

A concentrative tracer uptake assay developed by Garty et al. (1983) and modified by Goldberg and Miller (1991) was used to characterize the Cl\textsuperscript{−} and K\textsuperscript{+} transport properties of reconstituted pICln. pICln was reconstituted into liposomes without nystatin as described above for bilayer experiments. Proteoliposomes were preloaded with 150 mM KCl and external Cl\textsuperscript{−} was removed by centrifugation through Sephadex G-50 columns equilibrated with Cl\textsuperscript{−}-free uptake solution (125 mM K-glutamate, 25 mM Na-glutamate, 2 mM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 10 mM glutamic acid, and 20 mM Tris-glutamate, pH 7.6). Radioisotope uptake was initiated and quantified by addition of 1.0 μCi/ml of \textsuperscript{36}Cl\textsuperscript{−} to the proteoliposome suspension. Intravesicular \textsuperscript{36}Cl\textsuperscript{−} was assayed at various times after separation of proteoliposomes from the external media using a Dowex 1 mini anion-exchange column (Garty et al., 1983). To assess cation transport properties of pICln-containing proteoliposomes, a chemical gradient favoring cation efflux was generated by loading them with 150 mM KCl, and then exchanging the extracellular solution with a K\textsuperscript{+}-free uptake solution (150 mM NMDG-Cl, 2 mM CaCl\textsubscript{2}, 2 mM MgCl\textsubscript{2}, 10 mM glutamic acid, and 20 mM Tris-glutamate, pH 7.6). Electrogenic cation uptake was quantified by addition of 1.0 μCi/ml of \textsuperscript{38}K\textsuperscript{+} to the proteoliposome suspension.

In preliminary studies, we observed that half-maximal uptake of isotope occurred ~60 min after isotope addition. We reasoned that quantification of isotope uptake at 60 min would provide a sensitive measure of any differences in function between wild-type, phosphorylated, and mutant pICln. Accordingly, a 60-min uptake period was used in all isotope uptake studies.

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\textsuperscript{1}Abbreviations used in this paper: CKII, casein kinase II; GST, glutathione S-transferase; NMDG, N-methyl-d-glucamine.
Purification of Recombinant pICln

Recombinant pICln was produced in E. coli as a GST fusion protein and purified using glutathione Sepharose 4B and thrombin cleavage (see MATERIALS AND METHODS). For planar lipid bilayer studies, a control preparation was generated in parallel with the pICln preparation by expressing GST alone in E. coli. Lysates from GST-expressing bacteria were subjected to the same purification protocol (see MATERIALS AND METHODS) as lysates containing GST-pICln fusion protein.

Fig. 1 shows a Coomassie-stained gel and Western blot of the pICln preparation. pICln is the predominant protein detected in the purified bacterial lysates. When the gel is deliberately overloaded (i.e., 100 µg total protein), extremely faint protein bands were detected below pICln (Fig. 1 A). These proteins reacted with polyclonal anti-pICln antibodies (data not shown), suggesting that they were pICln truncation fragments. We also used N-terminus terminal sequence analysis to assess the purity of the preparation. pICln was the only protein detected using this method, which indicates that the preparation has been purified to at least 99% homogeneity.

pICln Generates Cation-selective Channels when Reconstituted into Planar Lipid Bilayers

pICln is a soluble cytoplasmic protein (Krapivinsky et al., 1994). In our initial attempts to reconstitute pICln, we added the protein in solution at a concentration of 50 µg/ml to the cis side of the bilayer chamber, an approach that has been used successfully to reconstitute channel-forming proteins such as VDAC (Xu and Colombini, 1997), bacterial toxins (Wimsen et al., 1990), and porins (Nekolla et al., 1994). Channel activity was detected using this method (e.g., Fig. 2 B), indicating that the channel-forming protein present in the purified bacterial lysates can spontaneously insert into the lipid bilayer. However, it was difficult to regulate protein incorporation into the bilayer with this approach. When protein incorporation occurred, multiple channels were usually detected. Single channels were more regularly detected by first reconstituting purified pICln into phospholipid liposomes before addition to the bilayer chamber. We also included nystatin and ergosterol into the liposomes to ensure that they were equally fusogenic, and to detect each fusion event as a transient increase in nystatin-induced bilayer conductance (see Woodbury and Miller, 1990). Fig. 2 C shows...
an example of liposome-to-bilayer fusion. The asterisks mark transient, nystatin-induced increases in conductance and indicate the fusion of a single liposome with the bilayer.

To assess whether the observed channel activity was specific to pICln or due to a minor contaminant present in the bacterial lysates, control liposomes were reconstituted with lysates purified from bacteria expressing GST only. Importantly, in >100 bilayer fusion events observed with the control liposomes, channel activity was never detected (e.g., Fig. 2 C). Channel activity was also never observed when lysates purified from bacteria expressing GST were added directly to the bath chamber (e.g., Fig. 2 A). In contrast, channel activity was consistently detected with the pICln-containing liposomes (Fig. 2 D). Channel activity was observed approximately every 10–20 liposome fusion events. Approximately 60% of the successful channel incorporations that occurred provided usable recordings (i.e., channel activity was stable and only a single channel was detected in the bilayer).

Fig. 2 E shows the current-to-voltage relationship of the channels reconstituted from the pICln-containing liposomes. With symmetrical 300-mM KCl solutions, the current displayed modest outward rectification and reversed close to 0 mV. The slope conductance measured between +10 and +60 mV was 48 pS. When the concentration of KCl in the trans bath chamber was reduced to 50 mM, the current reversal potential shifted from 1.1 ± 0.05 to −31.2 ± 0.06 mV (n = 3). The direction of the shift in reversal potential indicates that the channel is highly cation selective. Using the Goldman-Hodgkin-Katz equation, the calculated cation-to-anion permeability ratio of the channel is at least 7:1.

**Figure 2.** Channel activity detected in planar lipid bilayers reconstituted with pICln or lysates purified from bacteria expressing only GST. (A) Addition of lysates purified from bacteria expressing GST alone (see Materials and Methods) directly to the bilayer chamber has no effect on bilayer conductance. (B) Channel activity is observed when lysates purified from pICln-expressing bacteria are added directly to the bilayer bath chamber. These results indicate that the channel-forming protein can insert spontaneously into the lipid bilayer. (C) Example of liposome-bilayer fusion events detected as nystatin-induced transient increases in bilayer conductance (*). Control liposomes were reconstituted with proteins purified from bacteria expressing GST alone. Channel activity was never detected with control liposomes. (D) Example of channel activity detected after fusion of bilayer with liposomes reconstituted with pICln. (E) Current-to-voltage relationship of channels detected after fusion of bilayers with pICln liposomes. Slope conductance measured between +10 and +60 mV is 48 pS. Current is outwardly rectifying and reverses at 0 mV when both the cis and trans bath chambers contain 300 mM KCl. Reduction of KCl in the trans bath chamber shifts the reversal potential to −31 mV, demonstrating that the channel is highly cation selective. The cation-to-anion permeability ratio of the channel calculated using the Goldman-Hodgkin-Katz equation is at least 7:1.

**Cation Channel Activity Is Due to pICln and Not Contaminant Proteins**

The above results indicate that pICln functions in vitro as a highly cation-selective ion channel. However, it was possible that the channel activity observed was due to incorporation into the bilayer of a minor contaminant protein present in the purified bacterial isolate. To test for this, we assessed the ability of purified pICln reconstituted into liposomes to mediate concentrative, electrogenic 86Rb+ or 36Cl− flux. The flux assay described in

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the materials and methods section relies on the generation of an electrochemical gradient for cation or anion transport induced by the protein of interest. For example, if purified pICln is cation-selective, K\(^+\) should move down its electrochemical gradient out of the proteoliposomes when they are suspended in a K\(^+\)-free medium. Potassium efflux will in turn generate an intraliposome negative potential that will drive electrodiffusive uptake of \(^{86}\)Rb\(^+\) from the extracellular bath.

As shown in Fig. 4 A, pICln increased electrogenic \(^{86}\)Rb\(^+\) uptake approximately fourfold above that measured in liposomes without protein. In contrast, \(^{36}\)Cl\(^-\) uptake was unaffected by the presence of pICln in the liposome bilayer (Fig. 4 B). The results of the flux assay therefore support the bilayer studies and suggest that the observed cation-selective channel activity is due to pICln, which constitutes at least 99% of the protein in the preparation.

To further examine the possibility that a contaminant protein was responsible for the observed cation channel activity, we altered the biochemical properties of pICln and reconstituted the altered protein into planar lipid bilayers. pICln is phosphorylated by CKII (Sanchez-Olea et al., 1998). As shown in Fig. 5, phosphorylation of the protein significantly (\(P < 0.05\)) reduced channel open probability (\(P_o\)) from 0.82 ± 0.09 to 0.52 ± 0.11 (\(n = 5\)). Histograms of channel open and closed times for unphosphorylated pICln were fit by single exponentials with time constants (\(t\)) of 79 and 10 ms, respectively (Fig. 5 B). Phosphorylation of pICln had little effect on the channel open time constant, but it caused a decrease in channel open probability by inducing the appearance of a novel long closed state with a time constant of 207 ms (Fig. 5 B). These single channel data are consistent with the effect of phosphorylation on pICln-induced \(^{86}\)Rb\(^+\) flux. Reconstitution of phosphorylated pICln into liposomes reduced \(^{86}\)Rb\(^+\) uptake measured over a 60-min period \(\pm 50\%\) from 1,945 ± 558 to 920 ± 199 cpm (\(P < 0.05\); \(n = 3\); Fig. 5 C).

Paulmichl et al. (1992) presented compelling evidence to support the hypothesis that pICln is a anion...
channel-forming protein. These investigators identified the three glycine residues between G49 and G53 as being a possible nucleotide binding site. While this site has poor homology with known nucleotide binding motifs (Saraste et al., 1990), its location within the putative channel pore was consistent with the inhibition by extracellular nucleotides of the current induced by expression of pICln in oocytes (Paulmichl et al., 1992).

Paulmichl et al. (1992) mutated the three glycine residues to alanine (“AAA” mutant) and concluded that the mutant cRNA induced an anion current that was no longer inhibited by nucleotides. However, it has not been possible to reproduce these findings. Voets et al. (1997) have shown that expression of the AAA mutant induces a current identical to that of wild-type pICln. Furthermore, Buyse et al. (1997) have identified an endogenous current in oocytes with the characteristics of the current (Paulmichl et al., 1992) attributed to expression of the AAA mutant.

Because of its availability, we used the AAA mutant to ascertain whether it would have any effect on the basic biophysical properties of the reconstituted cation channel. As shown in Fig. 6, functional cation channels were reconstituted from the AAA mutant. However, the mutation significantly ($P < 0.05$) reduced channel $P_o$ from $0.82 \pm 0.09$ to $0.52 \pm 0.11$ ($n = 5$) by phosphorylation of pICln with CKII. Open and closed time histograms of channels observed for unphosphorylated pICln, and after phosphorylation with CKII (pICln + P), Histograms for unphosphorylated pICln were well fit by a single exponential ($\tau_{\text{open}}$, 79 ms; $\tau_{\text{closed}}$, 10 ms). Phosphorylation with CKII had no effect on channel open time ($\tau_{\text{open}} = 74$ ms), but it increased channel closed time. The closed time histogram was best fit by a double exponential. Both closed time constants were increased compared with unphosphorylated pICln ($\tau_1$, 28 ms; $\tau_2$, 207 ms). Reconstitution of CKII phosphorylated pICln into liposomes reduced $86\text{Rb}^\text{+}$ uptake ~50% ($^*P < 0.05$). Values are means ± SEM ($n = 3$). Isotope uptake was measured by incubating proteoliposomes with $86\text{Rb}^\text{+}$ for 60 min.

$pICln$ Increases the Cation Permeability Sf9 Cell Membranes

Data shown in Figs. 4–6 demonstrate that the cation channel activity observed in bilayers is due to pICln, and not minor contaminant proteins. To further examine this issue, and to ascertain whether pICln can function as a cation channel in biological membranes, we assessed the effect of pICln on the cation permeability of Sf9 cells.

As shown in Fig. 7, Sf9 cells shrank very slowly ($t_{1/2} = 9.8 \pm 1.4$ min, $n = 4$) when suspended in Na⁺- and K⁺-free medium (NMDG replacement) due to the loss of intracellular cations, Cl⁻, and osmotically obliged water. However, when the cationophore gramicidin is added to the bathing medium at a concentration of 10

\[ \text{Figure 5. Phosphorylation of} \quad \text{pICln with casein kinase II reduces} \quad \text{channel} \quad P_o \quad \text{and} \quad 86\text{Rb}^\text{+} \quad \text{uptake.} \quad (A) \quad \text{Mean channel} \quad P_o \quad \text{is reduced from} \quad 0.82 \pm 0.09 \quad \text{to} \quad 0.52 \pm 0.11 \quad (n = 5) \quad \text{by phosphorylation} \quad \text{of} \quad \text{pICln} \quad \text{with} \quad \text{CKII.} \quad (B) \quad \text{Open and closed time histograms of channels observed for unphosphorylated} \quad \text{pICln, and after phosphorylation with} \quad \text{CKII (pICln + P).} \quad \text{Histograms for unphosphorylated} \quad \text{pICln} \quad \text{were well fit by a single exponential (}\tau_{\text{open}}, 79 \text{ms;}\quad \tau_{\text{closed}}, 10 \text{ms}). \quad \text{Phosphorylation with} \quad \text{CKII had no effect on channel open time (}\tau_{\text{open}} \text{= 74 ms), but it increased channel closed time. The closed time histogram was best fit by a double exponential. Both closed time constants were increased compared with unphosphorylated pICln (}\tau_1, 28 \text{ms;}\quad \tau_2, 207 \text{ms}). \quad \text{(C) Reconstitution of CKII phosphorylated pICln into liposomes reduced} \quad 86\text{Rb}^\text{+} \quad \text{uptake ~50% (}^*P < 0.05\text{). Values are means ± SEM (}n = 3\text{). Isotope uptake was measured by incubating proteoliposomes with} \quad 86\text{Rb}^\text{+} \quad \text{for 60 min.} \]
μg/ml, the cells underwent a rapid shrinkage ($t_{1/2} = 0.52 \pm 0.18 \text{ min}$, $n = 4$). The fact that gramicidin decreases the halftime for shrinkage by ∼20-fold indicates that the resting cation conductance of the Sf9 cell membrane is low. Similar findings have been made by Vachon et al. (1995) using fluorescent probes to measure intracellular cation concentrations.

We reasoned that pICln should also induce rapid cell shrinkage if it functions as a cation-selective channel. When pICln was added to the bath at a concentration of 25 μg/ml, Sf9 cells shrank in a manner similar to that observed with gramicidin ($t_{1/2} = 0.54 \pm 0.12 \text{ min}$, $n = 4$; Fig. 7).

If pICln is responsible for the enhanced cation conductance of the Sf9 cells, then it should be possible to detect the protein in the plasma membrane. Fig. 8, A–D, shows immunofluorescence micrographs of native Sf9 cells and Sf9 cells treated with 100 μg/ml pICln for 60 min. Using an anti-pICln polyclonal antibody, we observed intense immunostaining of pICln in the plasma membrane of pICln-treated cells (Fig. 8, A and B). Importantly, immunostaining was not detected in non-pICln-treated cells (Fig. 8 D) or in pICln-treated cells exposed to preimmune serum (Fig. 8 C).

**Discussion**

When overexpressed in *Xenopus* oocytes, pICln induces an outwardly rectifying anion current that superficially resembles a ubiquitous swelling-activated anion current termed I_{Cl,swell} (Strange et al., 1996; Nilius et al., 1997; Strange, 1998). pICln was proposed initially to be the I_{Cl,swell} channel (Paulmichl et al., 1992; Gschwentner et al., 1995). We undertook reconstitution studies using recombinant pICln purified to at least 99% homogeneity to directly test the hypothesis that pICln is an anion channel–forming protein. As shown in Figs. 2 and 3, channel activity is observed in planar lipid bilayers after fusion with liposomes containing pICln. A crucial question that arises from these observations is whether the channel activity is due to pICln or minor contaminant protein(s) that copurify with it. We carried out several experiments to address this issue. Reconstitution of pICln into liposomes increased the ion permeability of the lipid membrane (Fig. 4). In addition, mutation or phosphorylation of pICln altered the gating properties of the channel and radioisotope flux (Figs. 5 and 6). Finally, when added to the external bathing medium, pICln incorporated into the plasma membrane of Sf9 cells (Fig. 8) and markedly increased membrane ion permeability (Fig. 7). These studies demonstrate clearly that pICln, and not contaminant proteins, is responsible for the observed channel activity.

The pICln channel has a high open probability, an intermediate conductance, and is outwardly rectifying. These characteristics superficially resemble those of the I_{Cl,swell} channel. However, the pICln channel is highly cation selective and has a relative cation permeability.
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(\(P_{\text{cation}}/P_{\text{anion}}\)) of at least 7:1. Consistent with this finding, the conductance of the pICln channel was undetectable when K\(^+\) was replaced by N-methyl-d-glucamine, but was unaffected by substitution of Cl\(^-\) with glutamate (Fig. 3). Furthermore, pICln increased the Rb\(^+\) but not the Cl\(^-\) permeability of liposomes (Fig. 4). In contrast to the high cation selectivity of the pICln channel, the IC\(_{\text{swell}}\) channel in mammalian cells has a \(P_{\text{cation}}/P_{\text{anion}}\) of \(\approx 0.03-0.04\) (Strange et al., 1996; Okada, 1997). We conclude, therefore, that pICln is not itself an anion channel-forming protein.

The cation selectivity of the pICln channel is consistent with the biochemical properties of the protein. pICln contains two highly acidic domains that are composed of multiple aspartate and glutamate residues (Krapivinsky et al., 1994; Emma et al., 1998; Sanchez-Olea et al., 1998). These domains almost certainly do not interact with the hydrophobic core of the lipid bilayer. If acidic amino acid residues line the channel pore, this would account for the cation selectivity of the pICln channel.

In addition to the present studies, several other findings have also challenged the hypothesis that pICln is the IC\(_{\text{swell}}\) channel. Voets et al. (1996) have shown that the pICln-induced current has characteristics distinct from those of IC\(_{\text{swell}}\) (Ackerman et al., 1994; Hand et al., 1997). Buyse et al. (1997) have shown that (a) expression in oocytes of an unrelated protein, CIC-6, induces the same current as that induced by expression of pICln, and (b) the pICln-associated current is observed without cRNA injection in \(\approx 5-6\%\) of oocytes (see also Paulmichl et al., 1992). Mutagenesis studies (Paulmichl et al., 1992) that provided compelling support for the hypothesis that pICln is a channel-forming protein have not been reproduced (Voets et al., 1997). Expression of AAA mutant pICln (Paulmichl et al., 1992) induces the same current as wild-type pICln (Voets et al., 1997). Furthermore, in native cells, it has not been possible to unequivocally detect pICln in cell membranes (reviewed by Strange et al., 1998). Based on these findings, it has been proposed that heterologous expression of pICln in oocytes activates an endogenous anion current distinct from IC\(_{\text{swell}}\) (Voets et al., 1996, 1997; Buyse et al., 1997; Strange, 1998). The anion current thought to be induced by expression of mutant pICln (Paulmichl et al., 1992) has also been proposed to arise from an endogenous oocyte anion channel (Voets et al., 1997).

Krapivinsky et al. (1994) proposed that pICln is a channel regulator and that expression of the protein in oocytes turns on an endogenous IC\(_{\text{swell}}\) channel. This hypothesis was based in part on the observation that the biochemical properties of pICln do not resemble those of typical ion channels, that the protein is localized predominantly to the cytoplasm, and that an anti-
pICln antibody inhibits swelling-induced activation of I_{Cl, swell} when injected into oocytes. Given the fact that the current induced in oocytes by pICln is distinct from I_{Cl, swell} (Voets et al., 1996), and that the same current can also be induced by the unrelated protein CIc-6 (Buyse et al., 1997), it seems unlikely that pICln is a regulator, in the strictest sense of the term, of the I_{Cl, swell} channel. It is clearly possible, however, that pICln indirectly affects I_{Cl, swell} activity. Disruption of pICln function by antibody injection into cells (Krapivinsky et al., 1994), antisense transfection (Gschwentner et al., 1995; Hubert et al., 1998), or overexpression (Hubert et al., 1998) may alter cytoskeletal structure, signal transduction pathways, etc., that ultimately affect I_{Cl, swell} activation.

What Is the Function of pICln?

The physiological function of pICln remains unknown and very controversial. We have suggested previously that pICln may function to regulate cytoskeletal properties and/or it may function as a “scaffolding protein” for bringing together and possibly regulating components of signal transduction pathways (Emma et al., 1998; Strange, 1998). Based on the results of these studies, it is clear that pICln can also function in vitro as a highly cation-selective channel. Does this imply that pICln has a physiological role as a cation channel? This question cannot be answered at present. pICln has not been detected unequivocally in the plasma membrane of native cells (reviewed by Strange, 1998), which argues that the protein does not have physiologically relevant channel activity. However, it is possible that small amounts of membrane-associated protein have escaped detection in previous studies, that pICln resides in cell membranes only under certain physiological conditions and/or that pICln may function as a channel in intracellular membranes. Further and more detailed studies of pICln–membrane interactions in vivo are clearly warranted.

It is possible that the pICln-induced cation channel activity may simply be a consequence of in vitro reconstitution of a recombinant protein. A number of proteins have been shown to generate channel activity in vitro, but have not been demonstrated unequivocally to do so in vivo. Annexins, for example, are a family of water soluble, calcium-binding proteins that are expressed abundantly in a wide variety of cell types (Kaetzel and Dedman, 1995). These proteins bind to plasma membranes and aggregate as trimers, hexamers, and multimers in response to increases in cell Ca^{2+}. The physiological function of annexins remains uncertain. They have been proposed to play roles in cell differentiation and mitogenesis, initiation of membrane fusion events important for exocytosis and endocytosis and inhibition of phospholipase A_2. Certain members of the annexin family also give rise to Ca^{2+} channel activity when reconstituted into planar lipid bilayers (Pollard et al., 1992; Luecke et al., 1995).

Conclusions

Our studies demonstrate directly that purified, recombinant pICln is capable of generating ion channel activity in both artificial and biological membranes. The channels are highly cation selective, a finding that argues strongly against the hypothesis that pICln is itself an anion channel–forming protein. Our findings provide a basis and justification for further physiological investigations of pICln. Structural studies of pICln may provide novel insights into the structure of channel-forming proteins and membrane–protein interactions. In addition, structural studies may provide insight into the native conformation of pICln, which, in turn, could provide clues about its physiological function.

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